

Impact of constitutional polymorphisms in immune effector mechanisms on clinical response to rituximab therapy in patients with follicular lymphoma

A submission for the degree of Doctor of Medicine
(Research)

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Contributions

The work presented here, unless stated otherwise, is the sole work of the author, Lisa Lowry.

The watch and wait trial was devised, set up and run by Dr Kirit Ardesbna, Professor David Linch, the trial management team and the staff of the CRUK & UCL Cancer Trials Centre (CTC). The central review of response assessments and other data cleansing was primarily undertaken by Lisa Lowry, with assistance from Kirit Ardesbna.

Trial statistics and generation of Kaplan-Meier curves was the work of Wendi Qian, MRC and CTC statistician. All other statistical analyses for this work were performed by the author.

The author undertook the majority of DNA extraction work and PCR analyses. Yashma Patel did some DNA and RNA extraction. Martin Pule, Yashma Patel, Alicia Kopec and Brian Phillips from UCL Cancer Institute taught the author the required techniques and providing general laboratory support. Martin Pule designed the PCR primers for *FCGR2B*-I232T detection. Direct sequencing was performed by staff at the UCL Cancer Institute.

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Abstract

The anti-CD20 monoclonal antibody, rituximab, has very significantly altered management and prognosis in patients with various B-cell malignancies, including follicular lymphoma. However, some patients respond less well to rituximab or become resistant to it. A better understanding of the mechanisms of action of rituximab in cancer patients and reasons for treatment failure could lead to optimal use of the drug and the design of more effective monoclonal antibodies in the future.

It has been suggested that constitutional polymorphisms in immune effector mechanisms may explain some inter-individual differences in rituximab effectiveness. Particularly, a polymorphism in the $\text{Fc}\gamma$ receptor IIIA has been found in several small studies to be correlated with clinical response to rituximab. A large, multicentre trial in newly diagnosed, advanced stage but asymptomatic follicular lymphoma randomised patients to receive up-front rituximab monotherapy or to watchful waiting. This cohort of patients provided an excellent opportunity to examine the possible effect of this, and other polymorphisms.

Patient samples were tested by PCR and restriction enzyme digestion for this polymorphism in $\text{Fc}\gamma$ receptor IIIA (*FCGR3A*-V158F), and linked with the cleaned clinical database. Other constitutional polymorphisms in $\text{Fc}\gamma$ receptors and the complement pathway have been suggested to play a role in rituximab response. Additional work determined polymorphism status for the same patient cohort for *FCGR2A*-H131R, *FCGR2B*-I232T, *C1QA*-Gly70_{GGG/GGA} and *C3*-R102G. In summary, there was no convincing evidence of a large effect of any of the polymorphisms studied. In particular, the *FCGR3A*-158V allele, considered by many to predict rituximab response on the basis of in vitro work and small, published series, was found to have no effect in this larger study. This has implications for future antibody design and usage, and interpretation of small pharmacogenetic response studies in general.

Introduction

Follicular lymphoma

Epidemiology

Non-Hodgkin lymphoma (NHL) comprises a group of heterogeneous malignancies of lymphocyte origin with distinct clinicopathological features. Various attempts at classification have been made in the past 5 decades; the World Health Organisation (WHO) classification published in 2001 and updated in 2008 has provided a reliable classification system, with around 50 subtypes of NHL identified.¹ NHL can be broadly categorised as aggressive or indolent based on clinical features and rate of progression; 80-90% are of B-cell origin with T-cell and natural killer (NK) cell-derived tumours being less common.

NHL is the 5th most common cancer in the UK with 12,793 people diagnosed in 2011, representing 4% of all cancer diagnoses.² Follicular NHL (FL) is the most common of the indolent lymphomas (although marginal zone lymphoma diagnoses have increased to similar levels) and the second most common type overall, representing 20-25% of NHL cases (the most common type being diffuse large B cell lymphoma (DLBCL)).² There are geographical and racial variations with the highest incidence reported in Northern America, Australasia and Northern Europe, and the lowest in South-Eastern Asia.³ In the United States, the incidence of B-cell lymphomas is higher in whites than blacks.⁴ The incidence of FL increases with age with a median age at diagnosis of 59 years. Unlike most NHL subtypes, there is a slight female preponderance with a female to male ratio of between 1.1 and 1.7:1.^{5,1} The cause and risk factors of FL are unclear, although environmental and occupational exposures, such as exposure to solvents and chemical, have been implicated in the aetiology of the disease.

Histology and pathogenesis

FL is a clonal proliferation of B cells of germinal centre origin.¹ Morphologically, the normal lymph node architecture is replaced by a neoplastic infiltrate exhibiting a follicular or nodular pattern reminiscent of germinal centres. FL can be graded histologically by the number of centroblasts and presence or absence of centrocytes.¹ Grade 1 and 2 FL has less than 15 centroblasts per high power field.

Grade 3 FL has more than 15 centroblasts per high power field; while centrocytes are still present in FL 3A, FL 3B is entirely composed of centroblasts. FL 3B is considered to be an aggressive lymphoma and patients are managed similarly to those with DLBCL.⁶

FL typically expresses B-lineage antigens including CD20, germinal centre markers CD10 and bcl-6, and bcl-2.¹ CD20 is a membrane-embedded, non-glycosylated phosphoprotein, virtually specifically found on B-cells and expressed in the majority of B-cell lymphoproliferative disorders (figure 1). It acts as a Ca^{2+} -permeable cation channel, involved in the regulation of B-cell activation, proliferation and differentiation.⁷ CD10 is a membrane-associated neutral endopeptidase expressed by a wide variety of normal human cells, including follicular centre cells. It is differentially expressed by neoplastic lymphocytes leading to its use in accurate lymphoma diagnosis.⁷ Bcl-6 (B-cell CLL/lymphoma 6) is a nuclear zinc finger transcription factor which functions as a transcriptional repressor and is necessary for germinal centre formation.⁷ It has been suggested that bcl-6 allows germinal centre B-cells to tolerate the physiologic DNA breaks required for immunoglobulin class switch recombination and somatic hypermutation without inducing a p53-dependent apoptotic response, by suppressing p53 expression. Deregulated bcl-6 expression may contribute to lymphomagenesis, in part, by functional inactivation of p53.⁸ Bcl-2 (B-cell leukaemia/lymphoma 2) is part of a family of pro- and anti-apoptotic proteins governing the permeability of the outer mitochondrial membrane and the release of cytochrome c to the cytoplasm.⁹ Bcl-2 is an inhibitor of apoptosis expressed normally on rapidly dividing cells, including T-cells, pro-B cells and mature B-cells. Bcl-2 is down regulated in normal germinal centre B-cells (where apoptosis forms part of the developmental pathway in order to select only cells producing antibodies with high avidity) but over-expressed in NHL and various other cancers.

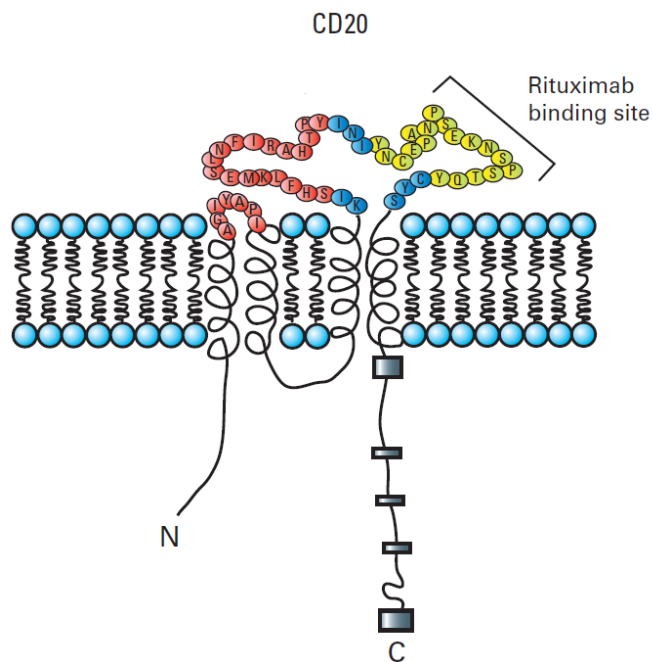


Figure 1: Schematic representation of a CD20 molecule. The larger of the two extracellular loops includes the rituximab-binding site. Figure from Cheson *et al*, 2010¹⁰

The chromosomal translocation $t(14;18)(q32.3;q21.3)$ is present in a large majority of patients and can be viewed as the initial genetic hit in the pathogenesis of FL.¹¹ Juxtaposition of the *BCL2* gene and the immunoglobulin heavy-chain (IgH) enhancer leads to deregulation of *BCL2* and constitutive expression of the Bcl-2 protein. Bcl-2 over-expression alone is insufficient for FL development; $t(14;18)$ has been detected at low levels in the peripheral blood of healthy individuals, the majority of which never develop overt follicular lymphoma.¹² Bcl-2 expressing FL cells fail to undergo apoptosis in the germinal centres and, therefore, are more likely to accumulate secondary chromosomal alterations and complete neoplastic transformation. The pathogenesis of $t(14;18)$ -negative FL remains largely unclear.

Natural history

FL is generally held to be an indolent but incurable malignancy with an inexorable drop-off seen on survival curves and a median overall survival (OS) of 7-10 years. The natural history is extremely variable with some patients remaining well for many years with minimal therapeutic intervention and others experiencing rapid progression and death despite aggressive therapy. Typically, patients relapse

after initial treatment but respond to further therapy, with recurrent progressions and progressively shorter remissions. Prior to the advent of rituximab, improvements in OS were already being seen in successive trials from the South West Oncology Group (SWOG), and in single-institution series, likely due to the availability of more therapeutic agents for treatment at relapse and improved supportive care (Figure 2).^{13,14,15} However, the anti-CD20 monoclonal antibody rituximab has revolutionised treatment in FL and other B-cell malignancies since the pivotal phase II study resulted, in 1997, in rituximab being the first FDA approved monoclonal antibody for the treatment of cancer.¹⁶ It is highly likely that further improvements in overall survival will be proven for those diagnosed in the rituximab era. However, there is still no curative treatment for most patients, many questions remain about the optimum timing and combinations of therapies, and not every patient responds to rituximab.

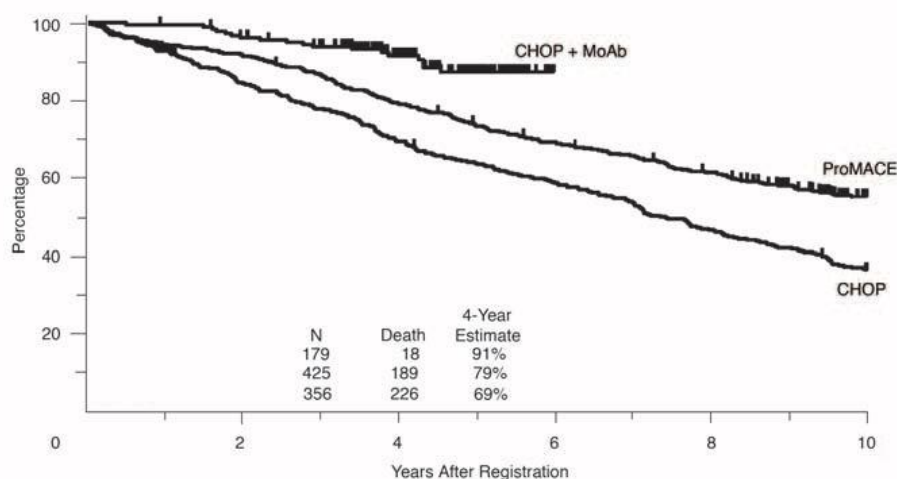


Figure 2: Overall survival in patients with advanced stage follicular lymphoma treated with multi-agent chemotherapy on South West Oncology Group (SWOG) trials in three different time periods. Successive improvements are seen between patients treated with CHOP (1974-1983), ProMACE (1988-1994) and CHOP + rituximab (1998-2000). Graph from Fisher *et al*, 2005¹³

FL most commonly presents with painless lymphadenopathy, which may wax and wane and even enter spontaneous remission. The majority of patients present with generalised adenopathy (stage III) and the bone marrow is involved (stage IV) in more than half, i.e. advanced stage disease.¹¹ Systemic (“B”) symptoms (fever,

night sweats, weight loss in excess of 10%) are infrequent at presentation but can be observed in later stages of the disease. Some patients present due to bulky intra-abdominal nodes which may compress adjacent structures. Primary mediastinal involvement and isolated splenic enlargement are uncommon. Transformation to an aggressive lymphoma is associated with a poor outcome; the rate of transformation varies in different series but is around 3% per year.¹⁷

Management

There is a wide range of treatment options in FL, from expectant management to allogeneic stem cell transplantation. Many chemotherapeutic and immunotherapeutic agents have efficacy in FL, which is also highly radio-sensitive. A curative treatment option is lacking, with the possible exception of allogeneic transplantation in a small minority of patients. However, most patients live with the disease for years, some for decades. Treatment therefore aims to maximise life expectancy whilst minimising treatment toxicity and optimising quality of life. There are many good quality published trials and yet many questions still remain about the optimum timing and combination of therapies. As new agents become available, choice in management will likely further broaden.

The choice of initial management is heavily dependent upon the clinical picture and patient co-morbidities and general health. Prognostic markers provide useful information but thus far have limited value in guiding initial treatment choice. The Follicular Lymphoma International Prognostic Index (FLIPI) stratifies patients into 3 groups with clearly different outcomes, based on the presence or absence of five adverse features: age 60 years or older, haemoglobin concentration less than 120 g/l, serum lactate dehydrogenase (LDH) above the upper limit of normal, stage III-IV, and greater than four involved nodal areas. The 5-year OS from the original publication prior to the availability of rituximab (retrospective data on patients diagnosed between 1985 and 1992) was 91%, 78% and 52%, in the low, intermediate and high-risk groups, respectively.¹⁸ Prognostic discrimination has been confirmed by a number of investigators in the rituximab era.^{19,20} More recently, the FLIPI2 was devised based on prospective data from patients treated in the rituximab era, combining the factors age, serum β 2-microglobulin, haemoglobin concentration, bone marrow involvement and tumour burden.²¹ The low, intermediate and high-risk groups included 20%, 53% and 27% of patients

and had 5 year OS of 98%, 88% and 77%, respectively. A group of patients with a very poor outcome, in whom more aggressive therapy might be warranted, cannot therefore be identified at diagnosis.

Management of limited stage disease

In the small proportion of patients presenting with limited stage disease (stage I and stage II with contiguous nodes), many consider radical radiotherapy to be the treatment of choice with the potential to cure around half the patients.^{22,23,24} The delivered dose of radiotherapy can be reduced to 24Gy from the previous standard 30-40Gy without compromising long-term outcomes;²⁵ in a recent trial comparing 24Gy with 4Gy more events were observed in the low-dose arm and such a reduction cannot be recommended in the setting of potentially curable disease.²⁶ The general consensus is that radiation fields can be limited to involved nodes with modest extension.²⁷ That said, observation alone might be suitable management and some series with careful patient selection suggest similar long-term outcome to radiotherapy; information from randomised trials is lacking.²⁸ There are also advocates of combined-modality therapy but data is limited for these approaches.²⁹ Patients with limited stage disease would, of course, be expected to respond to immunotherapy with rituximab but the long-term benefit is unknown. The idea of combining radiation therapy with rituximab is attractive as radiotherapy at low doses has been shown to increase CD20 expression on B cells in vitro.³⁰

Management of advanced stage disease

As the presentation and prognosis varies greatly for individual patients, so too do management options. A significant proportion of new patients are asymptomatic, having been diagnosed incidentally or following investigation of modest, untroublesome lymphadenopathy. Expectant management ("watchful waiting") has been employed in these patients with good long-term outcome; in a single-institution study of patients diagnosed over three decades ago the reported 10 year survival was 73%.³¹ Systemic therapy may still not be necessary in those with a single site of troublesome disease as radiotherapy can treat these areas very effectively, although not with curative intent in the setting of advanced disease. Initial watchful waiting has been cemented as the standard of care in newly diagnosed, asymptomatic patients with low-bulk disease by three randomised

trials comparing this approach with immediate chemotherapy with, respectively, single-agent chlorambucil, predimustine or interferon, and aggressive combined modality therapy.^{32,33,34} No difference was found in overall survival and patients were able to delay the initiation of therapy (with its attendant toxicities) by an average of 2-3 years (figure 3). Indeed, in the BNLI study, 19% had neither died of lymphoma nor required chemotherapy for it at 10 years, rising to 40% for those diagnosed aged 70 or more.³² With the advent of rituximab with its favourable toxicity profile, this question is being re-addressed and forms the basis of the trial from which the samples were obtained for this study (see page 51). Patients were managed with watchful waiting alone or with up-front single-agent rituximab; preliminary results have been presented showing a significant increase in progression free survival (PFS) and time to next treatment (TTNT) for those receiving rituximab.³⁵ Longer-term effects on responses to further lines of therapy and OS are not yet known and thus watchful waiting remains standard management, although early quality of life data may support the initial use of rituximab³⁶ and it is a reasonable treatment option.

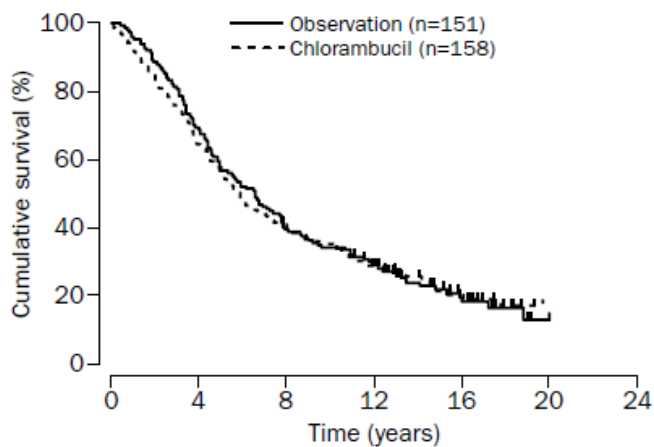


Figure 3: Overall survival in 309 patients with advanced stage, asymptomatic follicular lymphoma. Patients were treated on a randomised British National Lymphoma Investigation trial comparing initial observation (“watchful waiting”) with immediate systemic therapy with oral chlorambucil and overall survival was virtually identical. Graph from Ardeshtna *et al*, 2003³²

When treatment is required for advanced disease, the choice of approaches is wide, from single-agent regimens to combination immunochemotherapy, with the option of maintenance or consolidation therapy and the potential for autologous or allogeneic stem cell transplantation. There is good evidence that the initial regimen should include rituximab; several large randomised trials have shown significant improvements in response rates, PFS and, of great importance, OS using R-chemotherapy compared with chemotherapy alone.^{37,38} However, there is no gold standard chemotherapeutic regimen to combine with rituximab. Whilst differences may exist in response and PFS there is no evidence of improved OS for any first-line therapy. Patients are likely to require multiple lines of therapy over the course of their disease and therefore, ultimately, receive many different agents. In the UK, R-CVP (rituximab, cyclophosphamide, vincristine, prednisolone) or R-CHOP (R-CVP plus doxorubicin) are often viewed as standard first-line therapy. Prior to the advent of rituximab, there was no evidence that the use of intensive anthracycline-containing combination chemotherapy regimens conferred a survival advantage over single-agent alkylators.³⁹ In recent trials of immunochemotherapy there is a suggestion that patients who received anthracyclines had better PFS

than those who did not; however, this has not yet been compared in a randomised fashion^{37,38} Improvements in response must be balanced against the increased toxicity seen with anthracyclines as well as the inability to use them at a subsequent stage in the disease, such as at a time of high-grade transformation. Other first-line options include R-bendamustine, which has been shown to be highly effective with a more favourable side-effect profile than R-CHOP.⁴⁰ Fludarabine alone or in combination has achieved excellent results⁴¹ but is associated with increased risks of opportunistic infection and may impede mobilisation of stem cells for autologous transplantation at a later date. Rituximab monotherapy and the anti-CD20 radiolabelled monoclonal antibody ¹³¹I tositumomab (Bexxar) have been used as single agents in phase II trials with promising results.^{42,43}

It is possible to prolong remissions in those responding to induction therapy using rituximab maintenance, given every two or three months for 2 years. In the PRIMA trial, those achieving at least a partial response to first line therapy were randomised to rituximab maintenance or observation; with a median follow up of 3 years the PFS was significantly different at 75% and 58% respectively (figure 4).⁴⁴ As yet no difference has emerged in OS. The side-effect profile of rituximab is good compared with most chemotherapeutic agents; therefore the theory that more severe side-effects can be delayed is attractive. Fears of a high frequency of severe infections due to profound B-cell depletion have been shown to be unfounded. However, prolonged rituximab therapy is associated with an excess infection risk compared with observation following (immuno) chemotherapy induction. For example, in the PRIMA trial, the improved PFS in the maintenance arm came at the expense of a two-fold increase in infection risk, and a quadrupled risk of grade 3/4 infection (4% vs. 1%). Only one patient death in the 501 patients in the maintenance arm was felt to be possibly related to the treatment (fulminant hepatitis in the absence of antiviral therapy).⁴⁴ The number of cases of the fatal JC viral infection Progressive Multifocal Leukoencephalopathy (PML) attributable to rituximab is difficult to gauge, as haematological diseases are themselves a risk factor, but is in the order of 1 in 100000 patients treated.

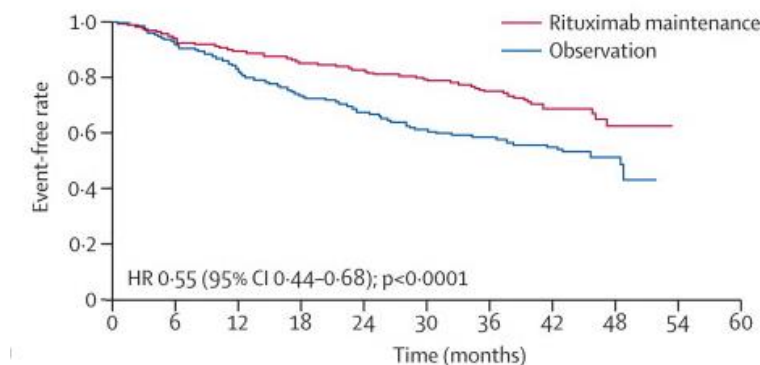


Figure 4: Progression-free survival on the PRIMA study. 1018 patients with advanced stage follicular lymphoma requiring up-front therapy, who responded to induction immunochemotherapy, were randomised to a 2-year period of rituximab maintenance or observation. The group allocated to maintenance therapy had significantly improved progression free survival. Graph from Morschauser *et al*, 2008⁴⁴

Similarly, the FIT trial randomised those responding to induction chemotherapy to observation or a single treatment with the radiolabelled monoclonal antibody ⁹⁰Y-Ibritumomab tiuxetan (Zevalin®). Again, significant improvement in PFS was seen with consolidation but not in OS.⁴⁵ The majority of patients on this trial did not receive rituximab as part of their induction as it predated its routine use, and it is not known if similar results would be achieved in patients treated with rituximab-containing induction regimens.

Treatment of relapsed disease

Although immunochemotherapy has improved outcomes, most patients are expected to relapse and require a succession of therapies over many years. The overall aim of therapy is similar to that at diagnosis – to optimise OS and quality of life. The options for management again include observation for those with low-bulk relapse, radiotherapy to particular sites of troublesome disease, radioimmunotherapy with ⁹⁰Y-Ibritumomab tiuxetan, and chemotherapy combined with rituximab. Many patients initially treated with rituximab have disease which remains sensitive to this agent after relapse. Rituximab maintenance can again be used after relapse in an attempt to prolong the period of remission,⁴⁶ although the degree of benefit from repeated periods of rituximab maintenance after successive relapses is unclear. Newer agents are being evaluated alone and in combinations,

including bortezomib and lenalidomide, bcl-2 inhibitors and drugs which target intracellular pathways such as spleen tyrosine kinase (Syk), Bruton's tyrosine kinase, the mammalian target of rapamycin (mTOR) and PI3-kinase. In the pre-rituximab era, high dose therapy with autologous stem cell rescue was of proven benefit in relapsed/refractory patients with chemosensitive disease who were fit enough to undergo the procedure.⁴⁷ The place of autografting has not been clearly defined in the rituximab era. Allogeneic stem cell transplantation holds the possibility of cure but myeloablative conditioning carries a transplant-related mortality (TRM) of around 30%. Whilst non-myeloablative conditioning has achieved encouraging results, the TRM is still at least 10%.⁴⁸

Some people will respond repeatedly to regimens containing rituximab and it is possible that in this way their disease can be managed as a chronic disorder over many decades. It is hoped that new antibodies will improve on the response rates and duration and offer alternatives in relapsing patients who have lost rituximab responsiveness. Much effort has gone into understanding the mechanisms of action of rituximab and the reasons for suboptimal response in patients in the hope that this will lead to the development of new antibodies in FL and, indeed, many other cancers.

Fc γ receptors

Antibodies contain Fab (fragment, antigen-binding) and Fc (fragment, crystallisable) domains. Activation of immune effector cells occurs after binding of their Fc receptors to antibodies via the Fc region; Fc γ receptors (Fc γ R) bind IgG antibodies. Fc γ Rs are members of the immunoglobulin superfamily. They have an IgG-binding α -chain with an extracellular portion composed of either two (Fc γ RII and Fc γ RIII) or three (Fc γ RI) Ig-like domains. Different classes of Fc γ R differ in cell distribution and affinity for IgG subclasses (table 1).

The FCGR gene cluster is located on the long arm of chromosome 1 (1q21-23) and consists of 8 genes (figure 5).⁴⁹ Fc γ RI is a high affinity receptor which binds monomeric IgG, coded by three genes (*FCGR1A*, *FCGR1B* and *FCGR1C*); however only Fc γ RIa is capable of binding IgG and the function of Fc γ RIb and Fc γ RIc is poorly characterized. Fc γ RII and Fc γ RIII are low-affinity, bind only complexed or multimeric IgG and are important in initiating ADCC. The Fc γ Rs can be classified by their effect, being either activating or inhibitory. The activating receptors contain an intracellular tyrosine-based activation motif (ITAM) for signal transduction. An ITAM is a specific sequence of amino acids (YXXL) occurring twice in close succession; phosphorylation of the tyrosine (Y) residue leads to a signalling cascade. Fc γ RIIb is an inhibitory receptor, containing an inhibitory motif (ITIM) in the cytosolic domain. Activating and inhibitory receptors are often co expressed on the same cell and the balance of the two signals determines the degree of cellular activation. Fc γ RIIIa binds only IgG1 and IgG3 isotypes; Fc γ RIIIa also binds IgG2. Polymorphisms in Fc γ Rs can influence the level of receptor expression, affinity for specific IgG isotypes or receptor function in terms of downstream signalling.⁴⁹

Several common SNPs and copy number variant regions exist at the FcR locus. Such genetic variation is characteristic of regions involved in immunity, likely because such diversity reduces the risk of population-destruction by a particular pathogen. This diversity has been very well described at the MHC locus. Fc γ R polymorphisms have been implicated in the risk and severity of a variety of infectious and autoimmune diseases.^{50,51,52} In very simplistic terms, FcR alleles

which somehow confer a lower threshold for activation would increase susceptibility to autoimmune disease but may provide for a better response to infection. Such SNPs may be postulated to enhance (or reduce inhibition of) response to monoclonal antibodies.

Receptor	FcγRIa	FcγRIIa	FcγRIIb	FcγRIIc	FcγRIIIa	FcγRIIIb
Alias	CD64	CD32	CD32	CD32	CD16a	CD16b
Gene	<i>FCGR1A</i>	<i>FCGR2A</i>	<i>FCGR2B</i>	<i>FCGR2C</i>	<i>FCGR3A</i>	<i>FCGR3B</i>
Expression	Macrophages monocytes neutrophils	Macrophages neutrophils platelets	Macrophages monocytes B-cells	Macrophages neutrophils	Macrophages monocytes NK cells	Neutrophils
Variant		H131R	I232T -386G>C -343G>C -120T>A	Q57X -386G>C -120T>A CNV	V158F	NA1/2
Effect			232T ↓ inhibitory activity -386C ↑ promoter activity Transcription al repression -120A ↑ promoter activity	Non- functional protein -386C ↑ promoter activity -120A ↑ promoter activity	158V ↑affinity IgG1 & IgG3	NA1 ↑affinity IgG1 & IgG3
IgG subclass specificity	3>1>4>>2	R131: 3>1>>2,4 H131:3>1,2>>4	3>1>4>>2		1,3>>2,4	1,3>>2,4

Table 1: The FcγR family. Encoding genes are shown, together with allelic variants with known functional impact^{49,53}

Rituximab mechanisms of action

Rituximab has become a mainstay in the therapy of B-cell malignancies, including FL, although its mechanisms of action are incompletely understood. There is evidence to support the importance of a variety of mechanisms; much of this evidence comes from *in vitro* work and *in vivo* murine models and may or may not relate to the situation in patients. Rituximab is not effective for all patients and various factors may contribute to clinical resistance to rituximab. Understanding the mechanisms of action of rituximab and reasons for variability in clinical response will hopefully lead to improvements in anti-CD20 monoclonal antibody therapy, and, indeed, inform the development of effective antibodies for use in a variety of cancers and other indications.

At the time of early interest in monoclonal antibody therapy for human cancers, CD20 was identified as an ideal target for such therapy. CD20 is found on most B-cell neoplasms, its expression is normally restricted to mature-B and pre-B cells, it is not shed from the cell surface, does not internalize upon antibody binding and is not found free in the circulation (thus an antibody would not be neutralized before binding to its target cell). CD20 is a 297 amino acid protein which spans the cell membrane four times. It is not known to have a natural ligand and its normal function is incompletely understood. Once the B-cell receptor (BCR) has been stimulated by antigen, it seems that CD20 collaborates and acts as a calcium channel.⁵⁴ CD20 knockout mice were found to be without obvious phenotype;⁵⁴ recently a case was reported of a girl with a homozygous mutation resulting in absence of CD20 expression. She presented with recurrent respiratory infections; although B cell development appeared normal she had impaired antibody responses.⁵⁵

Transient partial responses with few side effects were observed in lymphoma patients treated with a murine antibody to CD20.⁵⁶ Rituximab is a chimeric IgG1 antibody containing murine variable regions and human constant regions produced *in vitro* from a transfected Chinese hamster ovary cell line. The human constant regions allow for effective interaction with human complement and effector cells.⁵⁷ Potential mechanisms of action for rituximab include antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, complement-dependent cytotoxicity (CDC), and direct signalling leading to apoptosis. Other

suggested mechanisms include a vaccinal effect eliciting a T-cell response and complement-enhanced ADCC (CR3-ADCC). These mechanisms will now be discussed in turn; it is likely that more than one mechanism is at work and it may be that different mechanisms predominate in different clinical situations (figure 5).

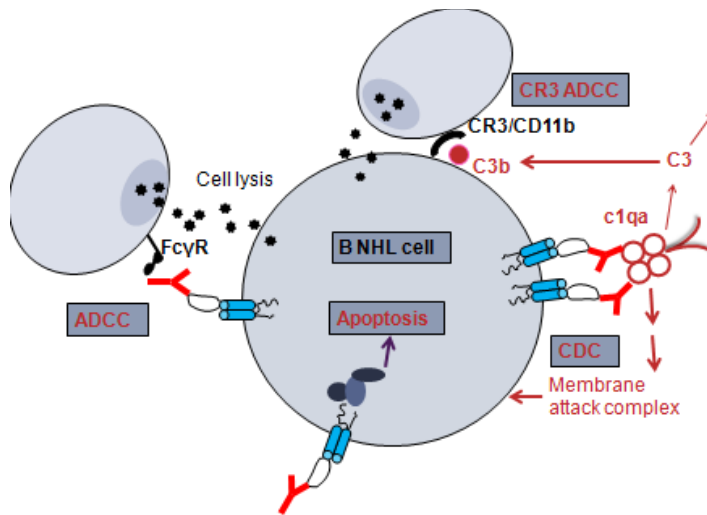


Figure 5: Possible mechanisms of action of rituximab. CD20 molecules are represented by twin blue cylinders with extracellular loop including rituximab binding site and intracellular domains. ADCC= Antibody dependent cellular cytotoxicity; CDC= Complement dependent cytotoxicity; CR3 ADCC= complement-enhanced ADCC.

Fc γ receptor-dependent mechanisms

Fc γ Rs are found on a variety of immune effector cells including natural killer (NK) cells and macrophages. ADCC depends on the activation of NK cells via the Fc-Fc γ R interaction leading to the release of substances toxic to the target cell such as perforin and granzyme, as well as cytokines which recruit other immune cells. Phagocytosis of pathogens is initiated by Fc γ Rs on macrophages binding antibody that is bound to antigen. Fc-Fc γ R interactions may also effect target cell death by cross linking antibodies leading to intracellular signalling and apoptosis.

ADCC

In vitro studies have used chromium release cytotoxicity assays to investigate ADCC in fresh lymphoma cells and lymphoma cell lines.^{57,58,59} Purified human effector cells cause tumour cell death in the presence of rituximab, with NK cells being the prominent effector cells in ADCC *in vitro*. However, *in vitro* detection of ADCC often involves much higher ratios of effector to target cells than would be found *in vivo*. Further evidence for the importance of Fc-Fc γ R interactions comes from murine models. Uchida *et al* demonstrated that mouse-anti mouse CD20 antibody depleted normal B cells *in vivo*, but not in mice lacking the FcR common γ chain, implying a critical role for Fc γ Rs. Furthermore, they showed that the depletion of B cells by rituximab was just as effective in mice with congenital deficiencies in the complement components C3, C4 or C1qa. B-cell depletion still occurred in mice with defective or deficient NK cells or T-cells but not in those rendered macrophage deficient, implying a central role for monocytes/macrophages, whether by ADCC or phagocytosis.⁵³ In a mouse lymphoma model, Clynes *et al* irradiated athymic nude mice and injected them subcutaneously with Raji human lymphoma cell line. They observed response to rituximab in wild type mice but not in those lacking the FcR common γ chain. In mice lacking the inhibitory Fc γ RIIb but with other Fc γ Rs intact, the response to rituximab was increased, providing evidence that the interaction of rituximab with a variety of Fc γ Rs on a variety of effector cells is central to determining the effectiveness of therapy (Fc γ RIIb not being expressed on NK cells).⁶⁰ They obtained similar results in mouse models of breast cancer treated with the anti-HER2 (Human Epidermal growth factor Receptor 2) antibody trastuzumab and melanoma treated with a murine IgG2 antibody TA99.

Rituximab infusion into lymphoma patients has been shown to result in NK cell activation,⁶¹ but the strongest evidence that ADCC is involved in clinical response comes from correlative studies linking outcome to Fc γ R polymorphism status. Fc γ RIIIa has either a phenylalanine (F) or a valine (V) at position 158. Fc γ RIIIa-158V has a higher affinity for IgG1 *in vitro* than Fc γ RIIIa-158F,^{62,63} and patients with at least one V allele have a greater degree of NK cell activation following rituximab infusion.⁶¹ Several studies have described significant improvements in response to rituximab and PFS in VV lymphoma patients compared to FF.^{64,65}

This has been interpreted as highlighting the importance of Fc-Fc γ R interactions in rituximab response and suggests that ADCC is of critical importance with NK cells, the principal cells expressing Fc γ R11a, being key contributors to *in vivo* response. The H131R polymorphism in Fc γ R11a has also been linked to outcomes following rituximab therapy.⁶⁵ However, not all studies have found these polymorphisms to be clinically relevant and those which did often included small patient numbers. The studies are discussed in further detail later in this introduction, when the individual polymorphisms are discussed.

Phagocytosis

A central role for macrophages which is Fc γ R dependent has been found in normal and malignant B cell depletion in murine models.^{66,67} Macrophages can phagocytose rituximab-opsonised cells *in vitro*.^{58,68} Another IgG1 monoclonal antibody, SGN35 (anti-CD30), has been found to cause FcR-dependent phagocytosis by macrophages *in vitro*, but not ADCC or CDC; response to SGN35 in murine models was lost if the mice were macrophage depleted but maintained if NK-cell depleted.⁶⁹ Leidl *et al* found that macrophages could phagocytose fresh B-Chronic Lymphocytic Leukaemia (CLL) cells (and also cells from FL and mantle cell lymphoma patients, but not cells from lymphoma cell lines) *in vitro* but could not induce ADCC.⁶⁸ They suggest that the principal way by which human macrophages contribute to rituximab response is by phagocytosis rather than ADCC, although the proportion of rituximab response attributable to macrophages in human patients rather than murine models is not clear.

Direct signalling

Rituximab can induce death of malignant B-cell lines *in vitro* in the absence of immune effector cells, although not all cell lines are sensitive and this has not been clearly demonstrated with primary lymphoma cells. Most researchers have found that detection of signalling changes requires hyper cross-linking of the rituximab with anti-rituximab antibody.^{70,71} The mitochondrial (intrinsic) pathway is reported to be employed in apoptosis induced *in vitro* by hyper cross-linked rituximab, with activation of caspase-9, cytochrome c release and mitochondrial disruption.⁷² Of interest, programmed cell death without such hyper cross-linking has been found to be independent of caspases.⁷¹ *In vitro* cross-linking of

rituximab may not reflect the situation in patients, providing such a strong signal that non-physiological changes are induced. Alternatively, cross-linking may occur *in vivo*, possibly again through Fc-Fc γ R interactions.

Several clinical observations suggest that rituximab can be effective against lymphoma cells without immune effector cells.⁷³ In an early trial of radiolabelled anti-CD20 therapy, patients were preloaded with unradiolabelled murine CD20 antibody IgG2a to improve biodistribution of the experimental therapy. Some subjects experienced reduction in lymphoma tumour burden after this pre-loading but before the radiolabelled antibody was given.⁷⁴ This suggests anti-tumour activity from the mouse antibody despite the fact that interaction between murine antibody and human Fc γ Rs is poor. Secondly, in a phase I study rituximab was administered directly into the cerebrospinal fluid of 10 patients with CNS involvement by B-cell lymphoma. Despite the lack of complement and immune effector cells in this compartment, 6 responded clinically, implying a direct effect of the drug.⁷⁵ Finally, the fact that rituximab plus conventional chemotherapy gives better clinical responses than either alone would suggest that immune effector cells are not critical to the response to combination therapy as they would be expected to be depleted by chemotherapy.

CDC

Rituximab can bind C1q *in vitro* and induce CDC of B cell lines and fresh lymphoma cells.^{57,76} The ability of a monoclonal antibody to fix C1q and lyse cells probably depends on the density of antigen and proximity to the cell membrane, as well as the particular binding site and spatial projection of the antibody. In addition, the ability of different anti-CD20 antibodies to reorganise CD20 molecules into lipid rafts is linked to their ability to cause CDC, presumably by clustering of available epitopes.⁷⁷ The level of expression of the complement-defence molecules CD55 and CD59 on tumour cells also contributes to the ability of rituximab to lyse cells by CDC *in vitro*.⁷⁶ Golay *et al* showed that sensitivity to rituximab-induced CDC of fresh CLL, MCL and PLL cells was dependent upon the level of CD20 expression, and also that of CD55 and CD59. Blocking complement-defence molecules in poorly responding CLL cells increased their sensitivity to lysis.⁷⁶ Manches *et al* also found that CD20, CD55 and CD59 expression on fresh lymphoma cells could predict *in vitro* CDC. They found that

different histological types of lymphoma were equally sensitive to ADCC, phagocytosis and apoptosis, but differentially lysed by CDC. CDC sensitivity was highest for FL, moderate for DLBCL and MCL and low for small lymphocytic lymphoma (SLL), suggesting that it is the degree of CDC which influences the *in vivo* responsiveness of different B-cell neoplasms to rituximab.⁵⁸ However, in a small study of 29 patients with FL, pre-treatment levels of complement-defence molecules did not correlate with clinical response to rituximab.⁷⁸

Clinical studies in patients with CLL have shown that complement is depleted following rituximab infusion,⁷⁹ and replacement by fresh frozen plasma (FFP) infusion might improve response. Animal model studies supporting a prominent role of CDC have used non-immunodeficient mice injected intravenously with murine lymphoma cells, engineered to express human CD20, and immunodeficient mice xenografted with human lymphoma cell lines.^{80,81} Mice rendered deficient in cellular effectors did not demonstrate any reduction in response to rituximab; however, complement depletion with cobra venom factor abolished it. The discrepancy with the results from the mouse models of Uchida *et al* and Clynes *et al* is intriguing.^{53,60} Uchida *et al* measured depletion of normal B cells and used murine anti-CD20 antibody, which may have important differences to rituximab, e.g. its ability to relocate CD20 antigens into lipid rafts. Golay *et al* acknowledge several possibilities which may explain the differences in their results compared with Clynes *et al*. Golay *et al* depleted NK cells and macrophages individually using antibodies or clodronate; ADCC may still be an effective mechanism if some cells remained after antibody/clodronate treatment or if the other cell type were able to compensate. However, they favour an explanation related to differences in tumour kinetics from the cell lines used. Xenograft models tend to have slow growing tumours, perhaps analogous to patients with bulky but indolent disease, whilst the cell line used by Golay is aggressive and grows rapidly. Rituximab therapy was shown to be effective against this rapidly growing lymphoma only up until day 15 from inoculation, around the time point of tumour detectability by PCR. The authors propose that this situation is analogous to that of minimal residual disease in aggressive lymphoma and suggest that CDC alone is sufficient for rituximab efficacy in this situation.⁸⁰

The formation of the membrane attack complex and cell lysis is one way in which the complement system could contribute to tumour cell death. Complement

components may also enhance phagocytosis by opsonisation, recruit effector cells chemotactically and enhance interactions between tumour and effector cells, thereby enhancing the action of rituximab by other mechanisms. In certain circumstances complement may be antagonistic to the effects of rituximab. C3b deposition on tumour cells *in vitro* seems to block Fc:FcγR interactions with NK cells, thus impairing ADCC.⁸² Complement has been implicated in enhancing “shaving”, a process by which CD20:rituximab complexes are removed from tumour cells, allowing them to escape further rituximab attack.⁷⁹ Finally, a SNP in the *C1QA* gene which leads to lower levels of C1q has been associated with prolonged clinical response to rituximab in FL patients, compared with the high-expressing allele (see below).⁸³

Vaccinal effect

Another proposed mechanism by which rituximab may exert some of its tumour control is via the generation of a tumour specific T-cell response. Regardless of the major mechanism of rituximab causing cell death, such cell death will liberate tumour antigens which can then be taken up and presented by dendritic cells to T-cells. Theoretically, rituximab could further favour the generation of such a cellular immune response via Fc:FcγR interactions with dendritic cells. Idiotypic-specific T-cells were found in 4 out of 5 FL patients one month after rituximab therapy, but not pre-treatment.⁸⁴ Another study suggesting a vaccinal role looked at a SNP at position 276 in *C1QA*. The polymorphism is non-coding with either a guanine or adenine as the third nucleotide in the codon for glycine at amino acid 70 (*C1QA*-Gly70^{GGG/GGA}). The G allele is associated with higher expression of the complement component C1q and increased CDC *in vitro*, compared with the A allele. The A allele has been reported to be over-represented in patients with subacute cutaneous lupus erythematosus.⁸⁵ The high-expressing allele might be expected to predict better rituximab responses if CDC were an important mechanism of action. However, the G allele was found to be linked with a shorter period of remission in FL patients who respond to rituximab. The authors of this study propose that high levels of C1q lead to opsinization of tumour apoptotic bodies and their efficient phagocytosis and removal, limiting the availability of tumour antigen for presentation to T-cells.⁸³

CR3-enhanced ADCC

During the classical complement cascade, C3b is generated from cleavage of C3 and binds to C3 convertase to form C5 convertase, leading to the formation of the membrane attack complex. C3b also acts as an opsonin and can interact with different receptors on immune cells, including complement receptor 3 (CR3) (also termed Mac-1 and CD11b/CD18) on NK cells, neutrophils and macrophages. Synergy of C3b:CR3 interactions with Fc:Fc γ R interactions have been suggested by *in vitro* studies.⁸⁶ In a melanoma mouse model, CR3 knockout mice were less protected by the monoclonal antibody TA99 than wild-type mice.⁸⁷ Imai *et al* studied EL4-lymphoma cell lines in a mouse model and used an IgG2 anti-GD2 antibody, which protected the mice from tumour progression and improved survival. Protection by the monoclonal antibody was not lessened in C3 or CR3 deficient mice, but almost entirely abrogated in mice deficient in Fc γ RI and Fc γ RIII, supporting a crucial role for ADCC. However, at low antibody levels, protection was partially lost in C3-deficient mice. The authors conclude that complement-mediated enhancement of ADCC was important at limiting antibody concentration.⁸⁸

Conclusions

A large body of literature now exists, providing evidence to support the central role of a variety of mechanisms of action of rituximab. Much of this, often conflicting, evidence comes from *in vitro* and mouse models and it is difficult to know how easily this transfers to the situation in human patients. In order to increase the body of evidence of clinical importance it is essential to push ahead with translational work linked to rigorously-run clinical trials. The strongest evidence of clinical importance is in favour of ADCC, from the correlative studies linking Fc γ R polymorphism status with clinical outcome. As previously mentioned, these studies were often small and underpowered, and not all reported the association. Nevertheless, they have directed a huge effort in therapeutic antibody development aimed at increasing affinity for Fc γ Rs and enhancing ADCC. The current research aimed to verify or refute these findings in a larger patient cohort and increase the body of evidence concerning rituximab mechanism of action in patients.

It is likely that ADCC, CDC, direct signaling and other mechanisms contribute to rituximab's effects in some patients at some time. Differences in tumour histology, bulk, and localization in the circulation or tissue probably affect the predominant mode of action in rituximab responders. In the next section, reasons for sub-optimal response to rituximab, and possible strategies to overcome them will be discussed.

Rituximab resistance

The term rituximab resistance has been widely used when discussing the range of tumour, patient and pharmacokinetic factors which may contribute to rituximab treatment failure. Few indolent lymphomas are truly rituximab-refractory with a decrease in tumour size of some degree seen in most patients after rituximab monotherapy.¹⁶

Lymphoma-related resistance

It is known that different histological types of B-cell malignancy have differing responses to rituximab monotherapy, with small lymphocytic lymphoma (SLL) and CLL having lower response rates than FL,^{16,89} and MCL having shorter event free survival than FL patients.⁹⁰ These differences may relate to the level of CD20 expression and that of complement-defence molecules as well as tumour kinetics and the predominant location of lymphoma cells (blood or tissues). Differences in active cell survival pathways and membrane lipid content may also contribute to different sensitivities.⁹¹

Patient factors

As previously discussed, the action of rituximab may depend on the availability and ability to utilise effector cells and complement components. Certain anatomical sites, for example the central nervous system, may lack certain effectors. Factors which could influence the number and activity of effector cells, and the availability of complement components in an individual could alter the observed therapeutic response. The subject of the current research, polymorphisms in FcγRs and complement components and receptors, are discussed more fully elsewhere. In addition, rituximab effector mechanisms could become saturated or exhausted under conditions of high tumour burden. Complement may be consumed for other reasons, such as infection or inflammation. Finally, as rituximab is not fully humanised it may stimulate formation of human anti-chimeric antibodies. This has been implicated in lack of response to rituximab in auto-immune conditions,^{92,93} although there is no evidence of a significant contribution to therapy resistance in lymphoma.^{16,94}

Rituximab concentration

Serum rituximab concentrations (and hence exposure) have been shown to correlate with clinical outcome, with higher concentrations seen in responders than non-responders.^{16,95} The original clinical studies of rituximab in NHL did not include dose-escalation to determine maximum efficacy or dose-limiting toxicities, rather the dose was rather arbitrarily chosen and this has become standard. Significant inter-individual variation in rituximab concentrations following the same dose has been noted;¹⁶ identifying the factors involved might enable more effective dosing. Rituximab pharmacokinetics are believed to be similar to that of normal immunoglobulin. In a study of 102 rheumatoid arthritis patients, the best model to describe the data was a two-compartment model with linear elimination pharmacokinetics.⁹⁶ Soon after the infusion the drug concentration reflected a distribution volume equal to that of plasma volume. There followed a biphasic decline in plasma antibody levels. Initially a rapid fall occurred, reflecting drug removal and distribution from the intravascular to the extravascular compartment, with a half-life of 2.4 days. During the later elimination phase the half-life was 19.7 days. In patients with FL, the observed half-life of rituximab increased from 3.2 days following the first of four weekly infusions, to 8.6 days following the last. Median maximum serum levels increased from 239 µg/ml following the first infusion to 460 µg/ml following the last.¹⁶ This can be explained using the same two-compartment model with different distribution and elimination half-lives. Trapping of rituximab by circulating CD20 positive B-cells following the first infusion may contribute. Rituximab is metabolised by non-specific catabolism in the liver and other organs, and excretion is predominantly renal.

In the study in patients with rheumatoid arthritis, gender and body surface area accounted for some of the inter-individual variability in rituximab concentration. Drug clearance was increased in men resulting in a 30% reduction in drug exposure compared to women.⁹⁶ There is a suggestion from the watch and wait study that men derive less benefit from rituximab maintenance than women (Dr Kirit Ardeshta personal communication). There may be interethnic pharmacokinetic differences as a Japanese study found lower serum rituximab levels in its patients than previously reported. They also found higher rituximab levels in patients without extranodal disease compared to those with extranodal disease.⁹⁷ The seminal study of rituximab in FL showed an inverse correlation

between rituximab serum concentrations and clinical response.¹⁶ The authors subsequently reported that baseline tumour burden and lymphocyte count correlated inversely with rituximab serum level.⁹⁵ The hypothesis that increasing tumour bulk results in a lower rituximab serum level and reduced clinical effectiveness due to increased antigenic mass could have significant implications for optimum dosing schedules. Work by Dayde *et al* in a murine model of disseminated lymphoma supports this hypothesis. When a fixed dose of rituximab was given, mice with a higher tumour burden achieved lower rituximab serum concentrations and had shorter survival. Increasing doses of rituximab were associated with improved response rates and survival, and through a pharmacokinetic / pharmacodynamic model the authors demonstrated that rituximab efficacy correlates with antigenic mass.⁹⁸ Lower serum concentrations of rituximab were also measured in the patients with SLL compared to other indolent lymphomas, and they had inferior responses, as previously noted. It is postulated that some of the reason for poorer response in SLL may be circulating tumour cells and antigen, which mops up available rituximab, lowering serum concentrations and decreasing response. Similarly, several studies reported low response rates in CLL when using the standard NHL dose of 375mg/m², but a dose-response effect can be demonstrated which probably relates to achieving higher drug concentrations.⁸⁹

Reduced or abnormal CD20 expression

A plausible explanation for antibody resistance would be mutations affecting the binding site. Several groups have investigated CD20 gene mutations and found no evidence of a significant contribution to rituximab resistance in DLBCL.^{99,100} CD20 expression may be down regulated following rituximab therapy, although there is no evidence that this is an important cause of rituximab-refractory disease. Chronic exposure of lymphoma cell lines to rituximab can lead to pre-transcriptional and post-transcriptional down-regulation of CD20.

“Shaving” of rituximab:CD20 complexes from the surface of circulating malignant B-cells has been implicated in sub-optimal response to rituximab therapy, particularly in CLL. It is suggested that, initially, circulating cells coated with rituximab are effectively killed by Fc:FcγR dependent mechanisms, whether ADCC or clearance by liver and splenic macrophages. However, if these mechanisms

become saturated, further rituximab-coated tumour cells have the antibody:antigen complex removed, and CLL cells with low CD20 expression can be detected.¹⁰¹

Strategies for overcoming rituximab resistance

Rituximab responses could theoretically be improved in three ways: optimising dosing, adding other factors to enhance effector mechanisms, or altering the antibody structure.

Optimising dosing

As previously discussed, the standard dose of rituximab employed was arrived at in a fairly arbitrary fashion. If rituximab is an effective treatment, then will more be better? Prolonging treatment with maintenance rituximab is of proven benefit after induction response in FL.^{35,44,102} Higher serum rituximab concentrations are correlated with improved clinical response. Simply increasing the dose to all patients will increase the average serum level; there is evidence of a dose-response effect in CLL.⁸⁹ Alternatively, concentrating doses more closely together will lead to faster drug accumulation and an earlier concentration plateau. In DLBCL, standard R-CHOP given every 14 or 21 days does not achieve a plateau in rituximab concentration until cycle 5 of therapy. The regimens DENSE-R-CHOP and SMARTE-R-CHOP combine increased dose-density early in the treatment course with either more rituximab doses altogether or a longer duration of therapy, respectively. Improved CR and EFS rates have been reported in around 100 elderly patients for each regimen compared with historical controls; interestingly it seems that the benefit may be restricted to those with poor prognostic features.^{103,104} The authors conclude that standard rituximab dosing may be sufficient for low-bulk disease whilst increased dose-density may be needed to optimise responses in bulkier disease. As serum concentrations may depend on disease bulk, histology and anatomical site, as well as patient gender and possibly race, optimising rituximab levels and therefore response may need to take all these factors into consideration. Alternatively, measurement of serum levels in clinical practice could identify patients who would be expected to benefit from increased dose or frequency of rituximab administration.

Increasing serum rituximab levels may be able to overcome any innate differences in patients' effector responses. An *in vitro* study of NK-mediated ADCC in Daudi

cell line found that 50% cell lysis was achieved by NK cells from *FCGR3A*-158V homozygous donors (encoding the higher affinity receptor) at a 4.2 times lower rituximab concentration than NK cells from *FCGR3A*-158F homozygotes. At saturating levels of rituximab, no difference was found in the degree of cell lysis, suggesting that any negative impact of the *FCGR3A*-158F allele *in vivo* might be overcome with increased serum levels. A mathematical model incorporating tumour mass, polymorphism status, dosing schedule and rituximab pharmacokinetics has been used to simulate plasma concentrations and linked with efficacy data to predict patient outcome. The model predicts improved PFS with higher doses of rituximab although this has not been tested prospectively.¹⁰⁵ Finally with respect to dosing regimens, it has been suggested that fractionated dosing may give better results in patients with circulating tumour cells. Small, frequent doses of antibody would avoid saturating effector mechanisms, implicated in the “shaving” reaction whereby tumour cells lose CD20 expression and thus become resistant to further doses of rituximab.¹⁰¹

Effector enhancement

Effector functions which contribute to rituximab mechanism of action could be augmented by co-treating with other factors. If CDC is an important mechanism and complement factors can be consumed, then replacement of complement with FFP may be a logical step. Complement activation can be enhanced by antibody to C3bi or oral β -glucans (which bind to CR3 and promote interaction with C3bi). If ADCC is of critical importance, expanding the effector cell number and increasing activation with cytokines such as IL-2, IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) might be beneficial. Either mechanism may be enhanced by up-regulating CD20 expression, which has been achieved *in vitro* with cytokines and a DNA methylation inhibitor.⁹¹ Information on efficacy and tolerability in humans of any of these possible approaches is limited or non-existent.

Antibody modification

The first generation of anti-CD20 monoclonal antibodies includes tositumomab as well as rituximab. So called second generation antibodies have a humanised or fully human complement-determining region, reducing the theoretical risk of

neutralising antibody development. Third generation antibodies have Fc modifications aimed at enhancing activation of effector cells or complement.

Considerable efforts have been made to develop next-generation anti-CD20 antibodies in the hope of improving response rates and providing options for patients who are rituximab-refractory. Such antibodies may have differing actions to rituximab if they either bind to a different CD20 epitope or have their Fc region engineered to enhance immune effector functions.

Antibodies may be divided into type I or type II, depending upon whether or not they relocate CD20 molecules into lipid rafts. Lipid rafts are cholesterol-rich membrane microdomains, which serve to compartmentalise cellular processes. Clustering of CD20 molecules in rafts by type I antibodies leads to efficient binding of C1q and CDC. Type II antibodies do not relocate CD20 to rafts and do not activate complement efficiently but are potent inducers of direct cell death. A list of CD20 antibodies is shown in Table 2; ofatumumab and obinutuzumab have the most clinical data available and will be discussed in more detail below.

	Type I	Type II
First generation	Rituximab	Tositumomab (B1)
Second generation	Ofatumumab Ocrelizumab Veltuzumab	
Third generation	Ocaratuzumab (AME-133) PRO 131921	Obinutuzumab (GA101)

Table 2: Therapeutic anti-CD20 monoclonal antibodies.

Ofatumumab

Ofatumumab's binding site is very close to the cell membrane, on the small extracellular loop of CD20 (rituximab binds to the large loop).¹⁰⁶ It is likely this proximity to the cell membrane that allows for more potent CDC than rituximab; it also has a much slower off-rate. Ofatumumab also causes more potent ADCC than rituximab in vitro; whilst both seem to be influenced by FcγRIIIa-V158F polymorphism in effector cells, both allotypes kill tumour cells more effectively with ofatumumab than rituximab.¹⁰⁷ However, responses could be improved with increasing concentrations of rituximab. Ofatumumab has not demonstrated

superior maximal efficacy (i.e. increased efficacy at the plateau of the dose-response curve) to rituximab *in vitro*, nor has any other type I antibody.

Ofatumumab was granted U.S. Food and Drug Administration (FDA) approval in 2009 for treating patients with CLL refractory to fludarabine and alemtuzumab, having produced an ORR of 58% when used as monotherapy in this hard to treat group.¹⁰⁸ It has efficacy in relapsed FL, including in a modest proportion of those resistant to rituximab.¹⁰⁹ Studies are ongoing in a variety of B-cell neoplasms including using ofatumumab monotherapy and various combination regimens; currently no results are available from randomised trials against rituximab and the UK National Institute for Health and Clinical Excellence (NICE) have not approved its use.

Obinutuzumab

Obinutuzumab is a glycoengineered, humanized, type II anti-CD20 antibody. Mossner *et al* (2010) describe the process by which it was created and selected for further study.¹¹⁰ Different humanized variants were produced from a parental murine antibody and tested for affinity to CD20 and ability to induce direct cell death in human B-cell lymphoma cells *in vitro*. Obinutuzumab demonstrated more potent binding to human CD20 compared to rituximab. Cellular assays demonstrated increased ADCC and direct cell death (and reduced CDC as expected in a type II antibody), and superior efficacy was demonstrated in animal models. However, higher concentrations or doses of rituximab were not evaluated to see if increased dosing could yield results comparable to obinutuzumab. Another study found obinutuzumab to be more efficient at inducing ADCC and direct cell death *in vitro*, compared to rituximab and ofatumumab, even at high antibody concentrations.¹¹¹ Animal models in this study again showed better tumour control with obinutuzumab compared with rituximab or ofatumumab, when similar dosing schedules were used.

Obinutuzumab has a sequence alteration in the elbow-hinge region.¹¹⁰ A valine residue is present at Kabat position 11, whilst the murine parent antibody had a leucine at this position. A variant with this residue remutated back to the original leucine displayed loss of activity with cellular responses similar to that seen with rituximab, despite maintaining the higher binding affinity of obinutuzumab.¹¹⁰

Obinutuzumab has also been found to have higher affinity binding to FcγR3b and associated increased activation of neutrophil phagocytosis *in vitro*.¹¹²

Obinutuzumab has shown clinical superiority over rituximab in a large, randomised trial in CLL. The CLL11 trial randomised 781 relatively unfit, treatment-naïve patients to chlorambucil alone, versus chlorambucil plus rituximab (R-chlorambucil), versus chlorambucil plus obinutuzumab (G-chlorambucil).¹¹³ Immunochemotherapy gave superior outcomes to chemotherapy alone. G-chlorambucil led to improved overall and complete remissions compared to R-chlorambucil, with improved median PFS of 26.7 months compared to 15.2 months ($p < 0.0001$). Of note, the doses of obinutuzumab used were higher than those of rituximab (3000mg vs. 375mg/m² in the first cycle, with 1000mg vs. 500mg/m² in subsequent cycles). The FDA approved its use in this setting in 2013, and guidance from NICE is awaited.

Polymorphisms chosen for study

FCGR3A-V158F

Fc γ R11a is a 29kD integral membrane glycoprotein encoded by the *FCGR3A* gene on chromosome 1. A SNP at nucleotide 559 (T-to-G) predicts a phenylalanine or valine at position 158, in the membrane-proximal IgG binding domain. The residue at position 158 directly interacts with the lower hinge region of IgG1.¹¹⁴ Allelic distribution shows little ethnic variation, with a minor allele (valine) frequency of 0.31 -0.37 and 9-16% homozygosity.^{64,115,116} *In vitro*, NK cells from *FCGR3A*-158V homozygous donors bind more IgG1 (and also IgG3 and IgG4) than do cells from *FCGR3A*-158F homozygotes (figure 6).^{62,63} This does not seem to be related to differences in levels of receptor expression between the genotypes.^{62,117} The number of circulating immune effector cells is also unrelated to polymorphism status.⁵⁹ Rituximab binding to NK cells from V-allele bearers is also significantly higher *in vitro* than F homozygotes, although the effect can be overcome with saturating rituximab concentrations (figure 7).^{118,117}

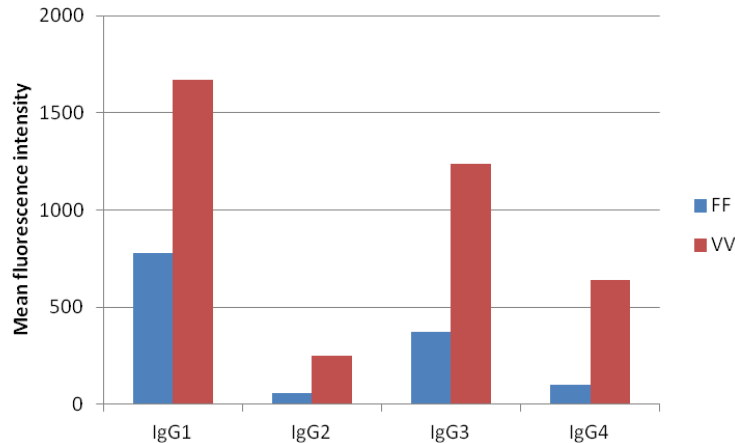


Figure 6: IgG binding by natural killer cells in vitro. NK cells from *FCGR3A*-158V homozygous donors bind significantly more IgG1, IgG3 and IgG4 than those from *FCGR3A*-158F homozygotes. Cells from heterozygotes exhibited intermediate binding, indicating a dose effect. Redrawn from Koene *et al*, 1997.⁶³

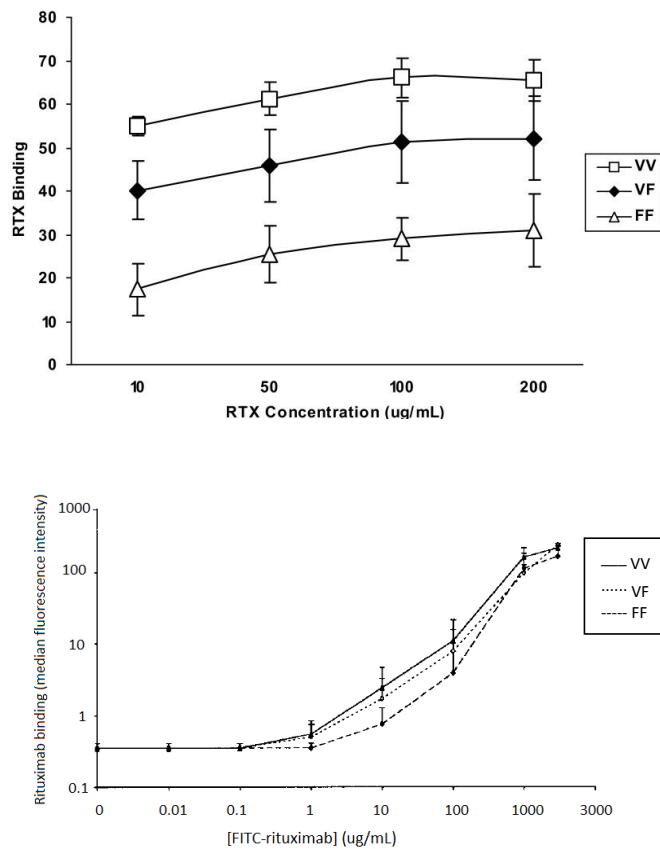


Figure 7: Rituximab binding at differing rituximab concentrations by NK cells from VV, VF and FF donors. NK cells were incubated with rituximab followed by FITC-labelled anti-CD16 antibody (graph from Hatjiharissi *et al*, 2007, top¹¹⁸) or with FITC-conjugated rituximab (graph from Congy-Jolivet *et al*, 2008, bottom¹¹⁷) and analysed by flow cytometry. Both sets of authors found that rituximab binding increased with increasing V allele dose in the range 10-100µg/ml rituximab; saturating doses of rituximab largely overcame the difference.

There is good evidence that the degree of NK cell activation by rituximab is influenced by *FCGR3A*-158F polymorphism. Stimulation of NK cells *in vitro* with the anti-CD16 monoclonal antibody 3G8 produced a brisk rise in intracellular Ca^{2+} whatever the genotype, whilst stimulation with aggregated IgG (still capable of cross-linking $\text{Fc}\gamma\text{RIIIa}$ molecules but presumably to a lesser degree) led to a 3-fold greater increase in intracellular Ca^{2+} in NK cells from V homozygous donors than F homozygous donors.⁶² In the presence of target cells (Raji Burkitt lymphoma cell line), higher rituximab concentrations were needed to produce increased CD54

expression on NK cells from F homozygotes than those from V homozygotes; the difference in activation was not statistically significant at saturating concentrations of rituximab.¹¹⁹ Similar findings have been reported *in vivo*. Blood was taken immediately pre-, and four hours post-, the first dose of rituximab in 21 patients with lymphoma. *FCGR3A*-158V allele bearers had a reduced percentage of NK cells in their circulating white cells, and increased CD54 and decreased CD16 expression on the NK cells, whilst *FCGR3A*-158F homozygotes did not.⁶¹

In vitro ADCC assays using purified NK cells against Daudi cell lines in the presence of rituximab have also shown increased cytotoxicity when using NK cells from *FCGR3A*-158V homozygous donors, either by chromium release assay with ⁵¹Cr- labelled Daudi cells⁵⁹ or colorimetric assay (figure 8).¹¹⁸ Again, there is evidence that the effect is overcome with saturating levels of rituximab, but the rituximab concentration resulting in 50% lysis (EC₅₀) of target cells was 4.2 times lower with V homozygous donors than F homozygotes.⁵⁹

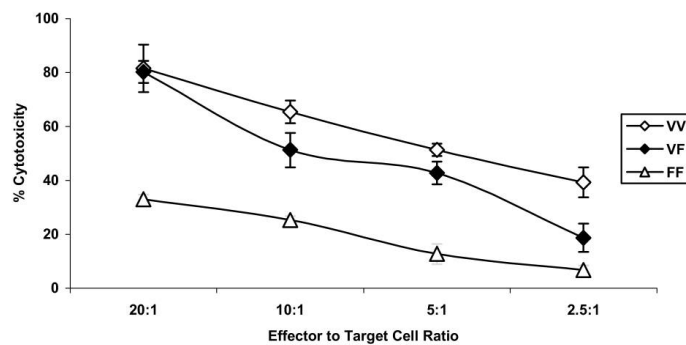


Figure 8: Increased natural killer cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) *in vitro* using *FCGR3A*-158V homozygous donors compared to *FCGR3A*-158F homozygotes; Cells from Daudi cell-line were incubated with 10µg/ml rituximab and NK cells from genotyped donors before cytotoxicity calculated using a colorimetric-based lactate dehydrogenase assay. Graph from Hatjiharissi *et al*, 2007.¹¹⁸

Whilst many *in vitro* and *in vivo* studies have reported functional differences between *FCGR3A*-158V homozygotes and 158F homozygotes, heterozygotes have been variously reported to be similar to either group of homozygotes, or in between. Differing levels of 158V protein expression have been documented amongst heterozygotes, with levels ranging from similar to V homozygotes to

similar to F homozygotes.¹²⁰ This heterogeneity may explain the range of results reported for heterozygotes.

These convincing *in vitro* demonstrations of correlation between *FCGR3A*-V158F polymorphism status and strength of IgG binding and effector cell activation prompted clinical investigation. In a landmark publication in 2002, Cartron *et al* reported 49 patients with low tumour burden, previously untreated FL, treated with rituximab induction alone.⁶⁴ Responses were indeed found to be associated with polymorphism status -100% and 90% response at 2 and 12 months respectively in VV patients, compared with 67% and 51% respectively in patients with at least one F allele. Subsequently this finding was replicated in other small studies; however, other publications have reported negative findings. Review of the literature is made more challenging by the use of different therapies to treat different types of B-cell malignancies in many of these reports. Table 3 summarises 29 publications in this area, published before or after our own work began. These studies generated conflicting results with some supporting the association with polymorphism status and others not finding this effect. Reports have involved different types of B-cell malignancies and different therapies, most involved small numbers and few are studies embedded were rigorous randomised controlled trials. We felt that the clinical correlation between response to rituximab and *FCGR3A*-V158F polymorphisms was not proven. The watch and wait trial offered an ideal opportunity to test the hypothesis in a larger patient cohort, rigorously followed up in a large, multicentre randomised controlled trial. In addition, the patients in this trial were rather similar to those in Cartron *et al*'s original publication,⁶⁴ being low tumour burden FL patients treated with rituximab alone. These patients would be expected to show the clearest influence of polymorphism status on rituximab response; patients with more aggressive disease or those treated with combination immunochemotherapy would likely owe less of their clinical response to rituximab, with subtle differences in response to rituximab less likely to be identified.

Study	Year	Diagnosis	Therapy	N	Part of RT?	Result
Cartron <i>et al</i> ⁶⁴	2002	FL	R-induction only	49	N	+
Weng & Levy ⁶⁵	2003	FL	R-induction only	87	N	+
Boettcher <i>et al</i> ¹²¹	2004	FL	R-CHOP+INF/ ASCT	75	Y – CLSG	-
Maloney <i>et al</i> ¹²²	2004	FL	CHOP then R	87	N	-
Farag <i>et al</i> ¹²³	2004	CLL	R-induction only	30	N	-
Treon <i>et al</i> ¹²⁴	2005	WM	R-monotherapy	58	N	+
Ghielmini <i>et al</i> ⁸⁰	2005			177		+
Pierz <i>et al</i> ¹²⁵	2010	FL	R+/- maint	151	Y – SAKK35/98	+
Martinelli <i>et al</i> ¹²⁶	2010			202		- *
Kim <i>et al</i> ¹²⁷	2006	DLBCL	R-CHOP	113	N	+
Carlotti <i>et al</i> ¹²⁸	2007	FL	CHOP then R	94	N	-
Mitrovic <i>et al</i> ¹²⁹	2007	DLBCL	R-CHOP	58	N	-
Galimberti <i>et al</i> ¹³⁰	2007	MCL	R-hyper CVAD	24	N	-
Dorman <i>et al</i> ¹³¹	2010	CLL	FC +/- R	419	Y - REACH	-
Zhang <i>et al</i> ¹³²	2010	DLBCL	R-CHOP	34	Y	+/-
Weng <i>et al</i> ¹³³	2010	Various;	HDT + ASCT	35	Y	-
Cornec <i>et al</i> ¹³⁴	2011	FL+MZL	R-induction only	50	N	+
Fernandez <i>et al</i> ¹³⁵	2011	FL	R maint post R-chemo	39	N	-
Ghesquires <i>et al</i> ¹³⁶	2011	DLBCL	R-CHOP	554	Y-GELA	-
Ahlgrimm <i>et al</i> ¹³⁷	2011	DLBCL	R-CHOP-14	263	Y-RiCOVER	-
Keane <i>et al</i> ¹³⁸	2011	DLBCL	R-CHOP	115	N	+/-
Fabisiewicz <i>et al</i> ¹³⁹	2011	DLBCL	R-CHOP	87	N	-
Prochazka <i>et al</i> ¹⁴⁰	2011	FL	Risk adapted	60	N	-
Treon <i>et al</i> ¹⁴¹	2011	WM	R-chemo	159	N	+
Persky <i>et al</i> ¹⁴²	2012	FL	mAB + chemo	76	Y - SWOG	+
Ghesquires <i>et al</i> ¹⁴³	2012	FL	R-chemo +/- maint	460	Y- PRIMA	-
Varoczy <i>et al</i> ¹⁴⁴	2012	DLBCL	R-CHOP	51	N	-
Chong <i>et al</i> ¹⁴⁵	2013	FL	R-lenalidomide	18	N	-
Tuscano <i>et al</i> ¹⁴⁶	2014	FL	R-lenalidomide	22	N	-
Zimmerman <i>et al</i> ¹⁴⁷	2014	PTLD	R-monotherapy	25	N	-

Table 3: Published reports of rituximab response by *FCGR3A*-V158F polymorphism status in patients with B-cell malignancies. FL=follicular lymphoma; DLBCL=Diffuse large B cell lymphoma; MCL=Mantle cell lymphoma; MZL=marginal zone lymphoma; PTLD=Post transplant lymphoproliferative disorder; R=rituximab; maint=maintenance. Positive results (+) reported more favourable clinical outcome in patients with V allele, negative results (-) found no association; +/- = non-statistically significant trend for benefit for V alleles.

*Three reports from a similar patient cohort in the SAKK35/98 trial. *FCGR3A*-V158F polymorphism status predicted outcome in earlier reports; however, with longer follow-up the predictive power was lost in multivariate analysis.

The *FCGR3A*-V158F polymorphism has been associated with risk and / or severity of various autoimmune diseases, which adds further circumstantial evidence for a

real clinical effect of enhanced Fc-FcR mediated immunity. The V allele has been linked with increased risk of childhood immune thrombocytopenia (ITP),¹⁴⁸ inflammatory myopathies,¹⁴⁹ atopy,¹⁵⁰ rheumatoid arthritis¹⁵¹ and clinically significant heparin-induced thrombocytopenia (HIT)¹⁵² and is associated with increased severity of Behçet's¹⁵³ and IgA nephropathy.¹⁵⁴

Improved response to rituximab in rheumatoid arthritis was reported to be associated with V allele carriage or homozygosity (in 111 and 212 patient studies)^{155, 156}, but not in a different cohort of 177 (heterozygotes responded significantly better than either group of homozygotes in this series).¹⁵⁷ Recently it has been reported that amongst 48 patients with ITP, responders were significantly more likely to be homozygous for the *FCGR3A*-158V allele.¹⁵⁸ An increased incidence of late-onset neutropenia following rituximab use in the treatment of B-NHL has been described following autologous transplantation¹³³ and first-line rituximab containing regimens.^{138,159}

FCGR2A-H131R

FcγRIIa is a 40kD glycoprotein encoded by the *FCGR2A* gene on chromosome 1. A SNP at nucleotide 519 (G-to-A) results in an arginine (R) to histidine (H) substitution at position 131 in the FcγRIIa molecule. A range of minor allele (R) frequencies have been reported, from 0.19 in Japan,¹¹⁵ to 0.38 in Ghana¹⁶⁰ and 0.48 in Spain and the Netherlands.^{116,161} The substitution is in the extracellular region, in the membrane-proximal Ig-like domain involved in binding of IgG. Whilst this region binds both IgG1 and IgG2, the specific loop bearing the polymorphism seems to play a greater role in IgG2 binding.¹⁶² The H131 variant has been shown to bind human IgG2-coated particles and lead to their phagocytosis in vitro.¹⁶³ The R131 variant, on the other hand, is much less capable of binding IgG2. Both variants bind IgG1 and IgG3; some authors have found the R allele to bind these subclasses more strongly.¹⁶⁴

Many associations between *FCGR2A*-H131R gene status and infectious, autoimmune and inflammatory conditions have been documented. The reasons for such associations seem to be more complex than is the case with *FCGR3A*-V158F, with susceptibility alleles for some infections and autoimmune conditions conferring protection against others. This may be linked to differential effect on avidity of binding of different IgG subclasses. Alternatively the explanation may lie

with differing modes of tissue damage in different inflammatory conditions.⁴⁹ Polymorphic variants leading to increased efficiency of IgG-Fc interactions may increase effector responses amplifying inflammatory cell-mediated tissue damage. Alternatively, they may reduce the chance of autoimmune pathologies related to circulating IgG complexes by enhancing clearance of such complexes. Finally, binding of other molecules may be affected by the polymorphism - FcγRIIa-131R has been found to bind C reactive protein (CRP) more avidly than FcγRIIa-131H *in vitro*, and to be associated with lower serum CRP levels *in vivo*.¹⁶⁵ This is likely to have interactions with risk of some inflammatory conditions, such as generation of cardiovascular atherosclerotic plaques. The R allele has been linked to increased risk of severe malaria,^{160,166} *Pseudomonas aeruginosa* infection in cystic fibrosis patients¹⁶⁷ and recurrent respiratory infections in Turkish children,¹⁶⁸ but decreased risk of pneumonia following H1N1 influenza infection¹⁶⁹ and decreased risk of bacteraemia¹⁷⁰ and death¹⁷¹ with pneumococcal pneumonia. *FCGR2A*-131R has been reported to be associated with increased risk of acute renal allograft rejection,¹⁷² myasthenia gravis,¹⁶¹ giant cell arteritis,¹¹⁶ heparin-induced thrombocytopenia¹⁷³ and atherosclerotic events.¹⁶⁵ On the other hand, *FCGR2A*-131H seems to be associated with risk of Guillain-Barré syndrome,¹⁷⁴ inflammatory bowel disease¹⁷⁵ and Kawasaki disease.¹⁷⁶ There are conflicting reports for several of these associations, possibly explained by genetic linkage with other important immune polymorphisms.

The *FCGR2A* -H131R polymorphism has been reported by some to predict rituximab response in patients with lymphoma,^{65, 177} whilst others have suggested that any association is due to wide linkage between *FCGR2A* and *FCGR3A*.^{178,179} Homozygosity for the H allele, which binds IgG2 more strongly, was reported to be associated with better responses and improved PFS in 87 FL patients, mostly relapsed, treated with rituximab monotherapy.⁶⁵ Another group found better responses but not improved survival in 64 patients with NHL of various subtypes treated with R-chemotherapy.¹⁷⁷ No association between polymorphism and outcomes were found in 58 patients treated first-line with R-CHOP chemotherapy for aggressive B-cell NHL,¹²⁹ 50 patients with indolent lymphoma treated with rituximab monotherapy,¹⁸⁰ 94 patients with newly-diagnosed FL treated with R-CHOP¹²⁸, 24 patients with mantle cell lymphoma treated with R-hyperCVAD¹³⁰ or 30 patients with CLL treated with rituximab monotherapy.¹²³ More recently, the PRIMA investigators reported no impact of this polymorphism on response to R-

chemo induction or maintenance rituximab in 460 FL patients, as part of a larger, randomised trial.¹⁴³ Improved PFS in *FCGR2A*-131H carriers was reported in 39 patients with previously treated, metastatic colorectal cancer treated with cetuximab,¹⁸¹ and 67 such patients treated with cetuximab in combination with irinotecan chemotherapy.¹⁸²

In view of the conflicting results generated by previous studies, and their small sample sizes, impact of the *FCGR2A* -H131R polymorphism on clinical outcomes was examined in our larger cohort of FL patients receiving rituximab.

FCGR2B-I232T

The inhibitory receptor FcγRIIb is a 34kD glycoprotein encoded by the *FCGR2B* gene on chromosome 1. It is the only FcγR found on B cells and cross-linking with the BCR leads to an increased threshold for cell activation and decreased antibody production. FcγRIIb is also found on other immune cells, when it inhibits the function of activating FcγRs. A T-to-C substitution in exon 5 of the gene leads to substitution of a polar threonine for a non-polar isoleucine at position 232 in the receptor transmembrane domain (figure 9). The functional consequences for the FcγRIIb-232T receptor are less efficient distribution of the receptor in lipid rafts, leading to reduced phosphorylation and reduced downstream inhibitory signal.¹⁸³

There is evidence linking the *FCGR2B*-232T allele to several autoimmune diseases, particularly SLE but also rheumatoid arthritis¹⁸⁴ and idiopathic thrombocytopaenic purpura (ITP)¹⁸⁵. The homozygous *FCGR2B*-232T allele is associated with an odds ratio of 1.73 for developing SLE.⁵¹ The frequency of the minor allele varies considerably from 0.1 (1% homozygosity) in Caucasian populations to 0.22-0.29 (5-11% homozygosity) in Africa and Southeast Asia,^{115,186} areas corresponding to endemic malaria. *FCGR2B*-232T homozygosity has been shown to confer substantial protection against severe malaria in a Kenyan population.⁵¹ This protective effect may account for the higher frequency of this SNP in Africans and Southeast Asians, which in turn may at least partially account for known ethnic differences in risk of SLE. A case-control genetic association study of *Cryptococcus* infection in HIV negative Chinese patients found over-representation of *FCGR2B*-232I homozygosity and under-representation of *FCGR2B*-232T homozygosity in the cases compared to controls.¹⁸⁷

One could propose that such attenuated inhibitory signalling could improve rituximab response in *FCGR2B*-232T allele bearers. A single previous study of 101 patients with FL did not find any influence on response to rituximab monotherapy.¹⁸⁸ Most of the patients in this study had had prior chemotherapy and only 2 were homozygous for the *FCGR2B*-232T allele with 15 heterozygotes. The potential association is re-examined in our larger cohort of FL patients. Recently, the level of expression of FcγRIIb was found to correlate with ORR and FFS in FL patients treated with rituximab monotherapy on the SAKK35/98 trial.¹⁸⁹ Stored, pre-treatment tumour blocks were stained with an anti- FcγRIIb antibody; 116 patients had absent or low-level expression and 13 had medium or high level expression. Those with medium/high expression had inferior ORR compared with those with negative/low levels (23.1% vs. 58.6%: $p=0.02$), and poorer FFS (2.8 months vs. 8.3 months: $p=0.002$).

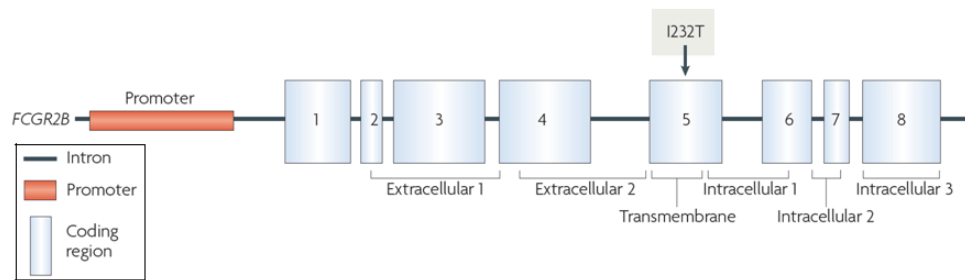


Figure 9: The location of the T-to-C single nucleotide polymorphism in exon 5 of the *FCGR2B* gene. Adapted from Smith and Clatworthy (2010)¹⁹⁰

C1QA-Gly70_{GGG/GGA}

The complement component C1q initiates the classical pathway of complement activation by binding to immune complexes, or non-immune complex activators such as lipopolysaccharide and outer membrane proteins of Gram-negative bacteria. C1q has also been shown to play an important role in the clearance of apoptotic bodies.¹⁹¹ The C1q molecule comprises 18 polypeptide chains, 6 A, 6 B, and 6 C chains, coded by three genes clustered on chromosome 1; *C1QA*, *C1QB* and *C1QC*.¹⁹² Patients with hereditary C1q deficiency are highly susceptible to recurrent infections and to systemic lupus erythematosus (SLE), and nonsense or missense point mutations have been identified in most of these familial pedigrees.

A transcriptionally silent polymorphism in exon 2 of *C1QA*, the gene encoding the α chain of C1q, has been identified, where the third guanine in the codon for amino-acid residue Gly70 is replaced by adenine (figure 10).⁸⁵ Polymorphism frequency in normal control populations are 13-18% GG, 21-29% AA and 53-66% heterozygotes, with A allelic frequency of 0.56.^{85,83} Even translationally silent mutations may influence gene expression;¹⁹³ alternative splicing of mRNA is a possible mechanism. The *C1QA*-Gly70_{GGA} allele is associated with lower levels of serum C1q and is strongly associated with a non-familial form of photosensitive skin disease, subacute cutaneous lupus erythematosus (SCLE).⁸⁵ When epidermal keratinocytes undergo ultraviolet light induced apoptosis, auto-antigens such as Ro are present in membrane blebs. The pathogenesis of cutaneous lupus is thought to include abnormal clearance of such auto-antigens. C1q binds to these membrane blebs and this binding is thought to lead to clearance of apoptotic keratinocytes.¹⁹¹ Patients with familial C1q deficiency have a 93% prevalence of photo-sensitive lupus.¹⁹⁴

Intriguingly, the *C1QA*-Gly70_{GGG/GGA} polymorphism has been reported to affect duration of rituximab response in FL.⁸³ In 133 FL patients treated with rituximab monotherapy, there was a trend for *C1QA*-Gly70_{GGA} allele carriers to have better responses than *C1QA*-Gly70_{GGG} homozygotes. Amongst those who responded, *C1QA*-Gly70_{GGA} carriers had a significantly greater duration of response, leading the authors to hypothesize that lower levels of C1q are associated with slower clearance of tumour apoptotic bodies, providing greater opportunity to develop a long-lasting immune response.⁸³ The same investigator group found the *C1QA*-Gly70_{GGA} allele to be associated with lower chance of metastasis in breast cancer patients, particularly haematogenous metastatic spread.¹⁹⁵ Impairment of apoptotic tumour cell removal and therefore prolonged exposure of various components of the immune system to tumour antigens may lead to a superior antitumor cellular response.

We decided to test for this polymorphism in our FL patient cohort in an attempt to confirm the findings of the single previous study.

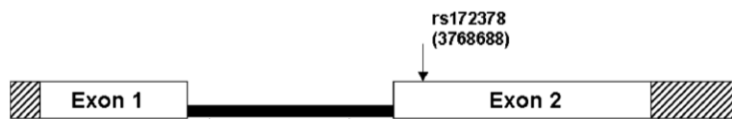


Fig. 10: The position of the C1QA-Gly70_{GGG/GGA} polymorphism. The intron is shown as a line, while the untranslated regions are represented by hatched areas. Adapted from Racila *et al*, 2006¹⁹⁵

C3-R102G

CR3 is a 187kD β 2-integrin which can bind multiple ligands including intracellular adhesion molecules, polysaccharides and iC3b, a cleavage product of C3.¹⁹⁶ It is encoded by the C3 gene on chromosome 19. Two allotypic forms of C3 have been described with a single-nucleotide polymorphism (C-to-G) in exon 3 at nucleotide 364 leading to either a positively charged arginine (R) or a neutral glycine (G) at amino-acid position 102.¹⁹⁷ This C3-R102G polymorphism is also called C3-S/F to refer to the slow or fast electrophoretic motility, and has also been referred to as C3-R80G or -Arg80Gly. The G allelic frequency is around 0.2 in Caucasians, less in black populations and extremely rare in Oriental populations.¹⁹⁸ Numerous disease associations reported with C3-R102G polymorphism argue for functional differences of the two alleles. It has reported associations with the autoimmune conditions IgA nephropathy¹⁹⁹ and systemic vasculitis²⁰⁰, presence of nephritic factor, an IgG autoantibody linked to mesangiocapillary glomerulonephritis and partial lipodystrophy,²⁰¹ and late-renal transplantation outcome.²⁰² Homozygosity for the C3-102G allele has been shown to confer an odds ratio of 2.6 for age-related macular degeneration (OR 1.7 in heterozygotes).²⁰³ There is also circumstantial evidence that the C3-102G allele may be advantageous in response to infection. Its prevalence is significantly higher in descendants of Dutch emigrants to Surinam than in the parent Dutch population, with a degree of difference unlikely to be accounted for solely by population drift.²⁰⁴ The emigrant population was subject to a 60% mortality from typhoid and yellow fever shortly after establishing the colony in the 19th century, strong selective pressure for polymorphisms enhancing recovery from infection.

The polymorphism was reported to affect the ability of C3 fragments to bind complement receptors²⁰⁵, although this has been called into question by subsequent work.²⁰⁶ The polymorphism is not thought to be located near to known

CR binding sites but in the MG1 domain near the binding site for factor H (fH) and its co-factor factor I (fI).²⁰⁷ Amplification of the alternative pathway of complement activation is controlled by fH and fI, which inactivate C3b. The C3-102G allele has been shown to reduce affinity for fH, leading to reduced C3b inactivation and increased complement activity.²⁰⁸ Reduced positive charge associated with the switch from Arginine to Glycine at position 102 is presumably responsible for the change in binding affinity.

In view of the associations with autoimmunity and infection, we decided to explore whether or not this polymorphism may have influence on the response to monoclonal antibody therapy. There have not been any previous such studies.

Patients, materials and methods

Study population

The study population comprised those patients who had received rituximab therapy on the intergroup randomised trial of rituximab versus a watch and wait strategy in FL. Consenting adult patients with newly diagnosed, asymptomatic, advanced stage FL (grades 1–2 & 3a) and adequate bone marrow reserve were included in the randomised trial (schema appendix 1). Trial inclusion and exclusion criteria, together with requirements for baseline assessment and randomisation procedures are listed in appendix 2. Between September 2004 and May 2009, 463 patients were randomised from 107 sites in 5 countries. They were randomly assigned to watchful waiting (arm A; n=187), rituximab 375mg/m² weekly for 4 weeks (arm B; n=84) or rituximab 375mg/m² weekly for 4 weeks followed by rituximab maintenance every 2 months for 2 years (arm C; n=192). Patients were randomised in a 1:1:1 ratio; arm B was closed part way through the trial to speed full accrual, as more information on the benefits of maintenance rituximab was emerging.

Patients were required to have low tumour burden, with a subtly different definition than that used by the GELF: the largest mass had to be less than 7cm with no more than 3 nodal sites greater than 3cm in diameter, the spleen no greater than 16cm on CT scan and no significant serous effusions. Additionally, haemoglobin had to be more than 10g/dl, neutrophils more than $1.5 \times 10^9/l$, platelets more than $100 \times 10^9/l$, and circulating tumour cells fewer than $5 \times 10^9/l$. Lactate dehydrogenase (LDH) was normal except in 5% of patients in whom it was slightly elevated. The primary clinical end point was time to initiation of next treatment (TTNT); secondary end-points included response at 25 months, cause specific survival and overall survival, as well as frequency of spontaneous remissions in arm A. The trial also included several quality of life end-points assessed by questionnaire. The trial protocol required provision of peripheral blood and bone marrow aspirate at registration and other time points (see below).

Ethical considerations

The randomised trial had appropriate regulatory and ethical approval and was conducted in accordance with the declaration of Helsinki. It was emphasized that participation was voluntary and that patients could refuse further participation at any time without prejudicing their subsequent care. Documented informed consent was obtained for all patients included in the study before they are registered at the data centre. Specific consent was sought for the storage and use of genetic material extracted from blood and bone marrow to correlate small differences in patients' immune systems with rituximab response, and to detect low levels of lymphoma. Patients also consented to the storage of genetic material to be used in future research into lymphoma pathogenesis and therapy response. Patients were able to participate in the clinical research study without agreeing to the use of their genetic material in this way.

In order to preserve confidentiality, patients' names were not recorded at the data centre; a sequential identification number was attributed to each patient registered in the trial. Patients' initials, dates of birth and local hospital numbers were also recorded to avoid identification errors.

Assessment of clinical response

Clinical response assessments were carried out by local investigators according to the criteria set out by Cheson et al (appendix 3)²⁰⁹ one month after randomisation and every 2 months thereafter for 2 years, then 3 monthly until progression. Once progression had occurred, follow-up continued 6 monthly. Immediate notification of disease progression, histological transformation, new treatment or death was required. CT scanning was mandated at 7 and 25 months, at 13 months if in clinical complete response (CR) and at any time to confirm progression or before new treatment. Bone marrow examinations were conducted at the same time points to confirm clinical and radiological CR if the marrow was involved prior to treatment.

Data quality was checked by data managers employing computerised and manual consistency checks on newly received case report forms; queries were issued in case of inconsistencies and/or missing data and followed up until resolution. Once

accrual was complete further database cleansing was undertaken. A random sample of patient files was physician-reviewed to ensure that accurate registration data had been recorded on the database. All reports pertaining to response assessments, disease progression including transformation, new treatment and patient death were physician-reviewed centrally, blinded to genotype results. Further queries were generated for clarification and in cases where the central reviewer disagreed with assessments performed at sites. In such cases the site principal investigator was invited to reconsider their assessment to reach consensus; if no consensus was reached the principal investigator's assessment was used.

Statistical analyses

The primary endpoint of the randomised trial was TTNT, defined as the time from randomisation until the first day of chemotherapy or radiotherapy administration. Progression free survival (PFS) was defined as time from randomisation to progression, transformation or death from any cause. Patients who are still alive are censored at the time they were last known to be alive. Overall survival (OS) was defined as time from randomisation to death from any cause. Patients still alive are censored at the time they were last known to be alive.

Overall response rates (ORR) and complete response rates (CRR) were analyzed by polymorphism and contingency tables created. CR and CR unconfirmed (CRu) were combined in all analyses. Fisher's exact test was used to derive p values from 2x2 contingency tables. The chi-square test was used to test for significant difference in contingency tables larger than 2x2. Where three patient groups are compared (homozygotes in either direction plus heterozygotes), p values derived from the chi-square test for independence (treating each group separately) and the chi-square test for trend are quoted. Kaplan-Meier survival curves for TTNT, PFS and OS were constructed; comparisons between groups were performed using the log-rank test. The product of the dimensions of the largest baseline node was calculated and patients were split at the median value and outcomes compared for those with lower bulk (LB) versus higher bulk (HB).

The sample size for the randomised trial was calculated to detect an improvement in the median TTNT in the rituximab arm of 18 months (a hazard ratio of 0.625) with a

5% significance level and 90% power, assuming a median TTNT of 30 months in the watch and wait arm. To observe the required 192 events, 180 patients were to be recruited into each of arms A and C with 2 years further follow-up after entry of the last patient.

Specimen collection and initial processing

Peripheral blood and aspirated marrow were collected at the time of registration, with repeat samples required at 7, 13 and 25 months if the patient was clinically and radiologically in CR. Blood and marrow were also required if a patient was due to start chemotherapy or radiotherapy. The protocol requested 20-30ml peripheral blood and the first 2-3ml aspirated marrow, each collected into ethylenediaminetetraacetic acid (EDTA), and stated that "samples should ideally be taken in the early part of the week (Monday to Wednesday preferably) and sent by first class post/guaranteed next day delivery if possible".

On arrival at the central laboratory, samples were registered by trial number, patient initials and date received, spun to isolate mononuclear cells and a white cell count performed. For those with adequate cell numbers, the sample was split to enable future ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) extraction. Samples for RNA extraction were stored at -80°C in 1ml trizol; those for DNA extraction were stored at -20°C in dodecyltrimethylammonium bromide (DTAB). Where cell numbers were very low, samples were only stored for RNA extraction. This initial processing had been carried out by various staff prior to the commencement of this project.

Australasian samples were received in frozen pellet form. Prior to DNA extraction they were defrosted at room temperature and re-suspended in 1.5ml DPBS. The suspension was transferred to a 15ml centrifuge tube, 3ml DTAB added and mixed by inversion. Samples were then frozen at -20°C and DNA extracted in batches (see below).

A list was compiled of patients from whom no adequate sample of blood or marrow had ever been received, who were randomised to receive rituximab. A letter was sent to principal investigators at sites requesting a fresh sample or stored material from blocks or slides (appendix 4). Due to concern that patients who had died on trial

were more likely to fall into this category, particular efforts were made to obtain stored material from them to reduce possible bias.

DNA isolation from blood or marrow

Genomic DNA was extracted in batches, using phase separation by centrifugation. Samples stored in DTAB were thawed to room temperature, mixed well with an equal volume of chloroform and centrifuged at 3500rpm for 15 minutes. The upper aqueous layer was pipetted into a clean tube and an equal volume of 100% ethanol added to precipitate the DNA. Further centrifugation at 3500rpm for 5 minutes formed the DNA into a pellet, which was removed to a 1.5ml eppendorf. The pellet was washed with 1ml 70-75% ethanol, centrifuged at 13000rpm for 1 minute, the supernatant removed and sample air-dried. The pellet was re-suspended in nuclease-free water, the volume of which was ascertained by the cell count of the original sample (<10 cells, 50µl water; 10-15 cells, 100µl; 15-20 cells 200µl, 20-40 cells, 300µl; >40 cells, 600µl). A cell count was not possible for Australasian samples; these pellets were re-suspended in 200µl nuclease-free water. Extracted DNA was stored at 4°C. DNA was quantified by spectrophotometer and samples diluted to approximately 250ng/µl for use in PCRs.

RNA extraction from blood or marrow

Samples frozen in trizol were defrosted at room temperature, 200µl chloroform added and shaken vigorously. Centrifugation at 12000rpm for 15min at 4°C resulted in separation into an organic phase and an aqueous phase containing the RNA. The upper aqueous layer was transferred to a fresh tube and 0.5 ml isopropyl alcohol added to precipitate RNA. Samples were incubated at room temperature for 10 minutes then centrifuged for 10 minutes at 12000rpm and 4°C. The supernatant was discarded, 1ml cold 75% ethanol added and sample centrifuged for 5 minutes at 7500rpm and 4°C. The supernatant was carefully removed leaving the RNA pellet undisturbed. The pellet was air-dried at room temperature and re-suspended in nuclease-free water; the sample was stored at -80°C.

Alternative DNA sources

Where no genomic DNA was available, complementary DNA (cDNA) was prepared from RNA using Invitrogen Superscript III kit. RNA was defrosted and quantified by spectrophotometer. Working on ice, 1µg RNA was made up to 10µl with water in PCR tubes before adding 2µl random decamers (Ambion) and 4µl 2mM deoxynucleoside triphosphates (dNTPs). Samples were heated to 70°C for 3 minute and returned to ice for 1 minute. Reverse transcriptase, 10xRT buffer and RNAs inhibitor (1µl of each) were added prior to incubation at 42°C for one hour then 95°C for 5 minutes. Samples were refrigerated for use within the next few days or frozen at -20°C for future use.

Where no peripheral blood or bone marrow aspirate was available, DNA was recovered from stained, glass slides. They were soaked overnight in xylene to facilitate coverslip removal. Each slide had 20µl proteinase K and 200µl phosphate buffered saline (PBS) added and the material scraped into a 1.5ml eppendorf tube. Following the addition of 200µl AL buffer the samples were incubated at 56°C for 5 hours to achieve cell lysis. 200µl absolute ethanol was added before transfer to a DNeasy mini spin column and centrifugation at 8000rpm for 1 minute; flow-through was discarded. 500µl buffer AW1 was added and the column centrifuged at 8000rpm for 1 minute with flow-through discarded; 500µl buffer AW2 was added and spun at 14000rpm for 3 minutes. The column was placed in a new 1.5ml eppendorf tube with 100µl buffer AE, kept at room temperature for 5 minutes before centrifugation at 8000rpm for 1 minute. A second aliquot of buffer AE was added and further spun into the receiving eppendorf.

Analyses for polymorphisms by polymerase chain reaction (PCR) and allele-specific restriction enzyme digestion

General PCR methods

Primers were synthesized and lyophilized by Integrated DNA Technologies. On receipt in the laboratory they were reconstituted with nuclease-free water to 100µM stock solutions and frozen at -20°C. Prior to use they were thawed at room temperature, diluted to 25µM in nuclease-free water, and re-frozen in aliquots for

each PCR batch to minimise the potential for contamination. Each PCR reaction was carried out in a 50µl volume containing 1µl of each primer at this working dilution (25pmol), 25µl *Taq* 2x mastermix (New Eng biolabs: 25µl contains 1.25units *Taq* DNA polymerase, 0.4mM dNTPs, standard *Taq* reaction buffer and stabilisers), 21µl nuclease-free water and 2µl of each DNA sample (approximately 500ng). Larger sample volumes were used in cases of very low DNA concentration, and less water added.

A blank control was carried out in parallel for each batch, replacing the DNA sample with water. Great care was taken with labelling of the tubes and pipetting patient samples to ensure correct identification. PCR reactions were performed on a DNA Engine Tetrad 2 thermal cycler (Bio-rad).

FCGR3A-V158F polymorphism detection

The *FCGR3A*-V158F polymorphism was ascertained by nested PCR and restriction enzyme digestion based on a published method.⁶³ The *FCGR3A* gene sequence, location of the SNP and sites of primer annealing were identified (appendix 5). The first PCR reaction used outer primers (forward MP6965: 5'-ATA TTT ACA GAA TGG CAC AGG -3' and reverse MP6966 5'-GAC TTG GTA CCC AGG TTGA A-3'; characters in bold denote mismatches introduced to increase specificity) to amplify a 1.7kb fragment containing the polymorphic site. Patient samples were processed in batches of 18 in 50µl reaction volumes (see above). Initial denaturation for 10 minutes at 95°C was followed by 35 cycles of denaturing at 95°C for 1 minute, primer annealing at 56°C for 90seconds and extension at 72°C for 90seconds, followed by a final extension of 8minutes at 72°C.

The second PCR used inner primers (forward MP6967: 5'-atc aga ttc gAT CCT ACT TCT GCA GGG GGC AT-3' and reverse MP6968: 5'-acg tgc tga gCT TGA GTG ATG GTG ATG TTC AC-3'; capital letters denote annealing nucleotides) to amplify a 94bp fragment. The forward primer contained a mismatch (A instead of T two nucleotides from the 3' end), which created an *Nla*III restriction site (5'...CATG⁺...3') in *FcγR3A*-158V encoding DNA but not *FcγR3A*-158F. 2µl of the first PCR product was used as the template for this second PCR. Reaction conditions were 5 minutes denaturing at 95°C, 1 minute annealing at 64°C and 1 minute extension at 72°C for

the first cycle, followed by 35 cycles in which the denaturing time was 1 minute, followed by a final 9½ minute extension at 72°C. The DNA product was transferred to a QIA quick spin column for purification. Binding buffer PB was added at five times volume (250µl) and spun for 1 minute with through-flow discarded. 750µl wash buffer PE was added and centrifuged for 1 minute; flow-through was discarded and the samples spun for a further minute. The DNA was then eluted into 30µl nuclease-free water.

Digestion was carried out in 50µl reaction volumes using 10µl DNA and 2µl *Nla*III enzyme (New England Biolabs) with the recommended buffer, incubated at 37°C for 2 hours. In FcγR3A-158V encoding DNA, the 94bp fragment was cut into two fragments of 61bp and 33bp, whereas FcγR3A-158F encoding DNA remained undigested. Previously identified FF, VV and VF samples were included in each batch as a control for adequate digestion. Following digestion and the addition of 10µl DNA load, samples were run on 3.5% agarose gels containing gelstar, viewed under UV light and the genotype recorded. Sample gels are shown in figure 11.

The product of the second PCR was too short to generate a good quality sequencing result. The product of the first PCR from homozygous F, homozygous V and heterozygous patient samples was sequenced using the forward primer to corroborate the results (figures 12 and 13).

Assay optimisation:

- 1) Work was required to optimize the staining of the gels to pick up small fragments. Gels were pre- or post-stained with gelstar or ethidium bromide. Pre-staining with either stain produced an extra non-specific band, slightly larger than the 61bp fragment, not seen on post-stained gels (figure 1C). However, pre-staining with gelstar gave the sharpest band appearance and hence this method was used for patient samples (3.15g agarose melted in 90ml TBE with 9µl gelstar added).
- 2) Initially there were problems with contamination (product in the blank control) despite carrying out the assay in a room reserved for PCR using techniques standard in the laboratory. This was largely solved by reserving a set of new pipettes and separate tips for this work.

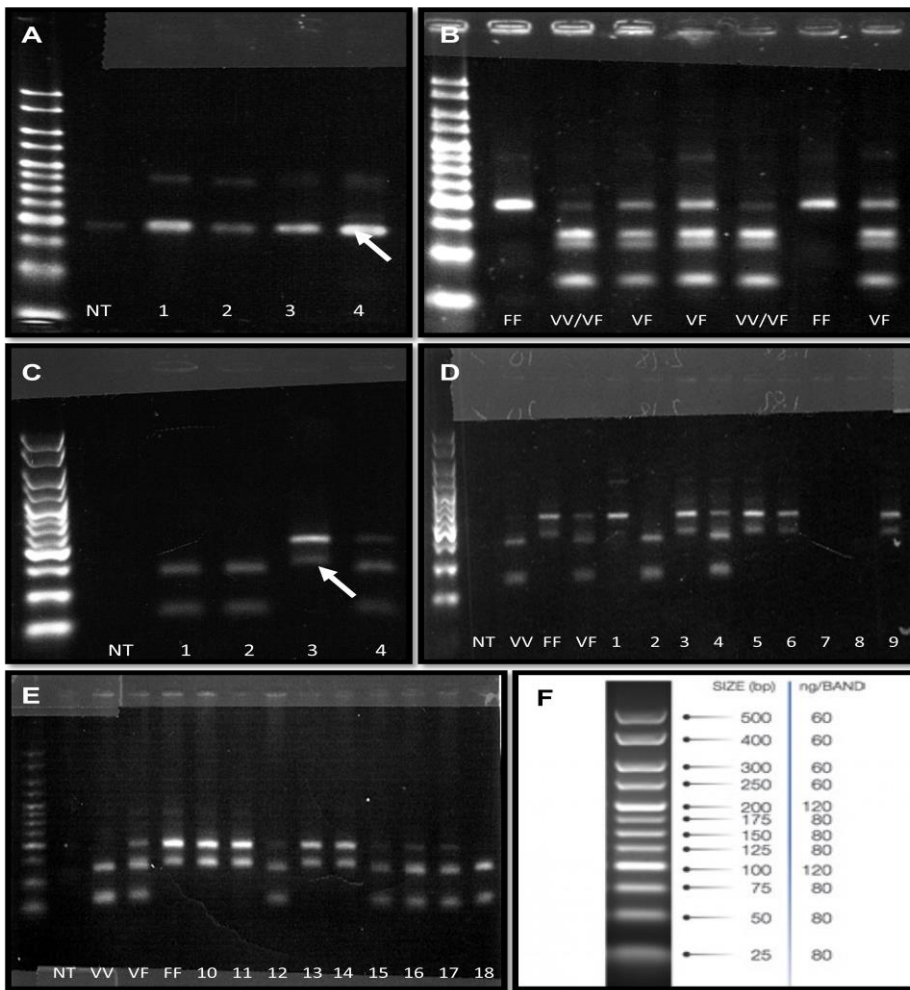


Figure 11: *FCGR3A*-V158F polymorphism analysis: high-resolution agarose gel photographed under UV light post electrophoresis. A) 94bp undigested product (arrowed) in screening samples 1-4 (also present in blank control) B) Post digestion bands of 94bp, 61bp and 33bp are seen, depending on genotype. This gel was post-stained with gelstar. Homozygous F samples (uncut) are easily identifiable but the difference between homozygous V and heterozygous is less clear-cut. C) Digested screening samples run on gels containing gelstar. A non-specific band is seen relating to the F allele (arrowed) but the VV and VF samples can now be well differentiated. D), E) Patient samples following nested PCR, restriction digest and electrophoresis on 3.5% high resolution agarose gels containing gelstar, viewed under UV light. Patients 1, 3, 5, 6, 9, 10, 11, 13 and 14 are homozygous F; patients 4, 12, 15, 16 and 17 are heterozygous; patients 2 and 18 are homozygous V. No product was identified for patients 7 and 8 and these were repeated. F) Hyperladder V used in the first lane in gels A-E. NT = No template (blank control)

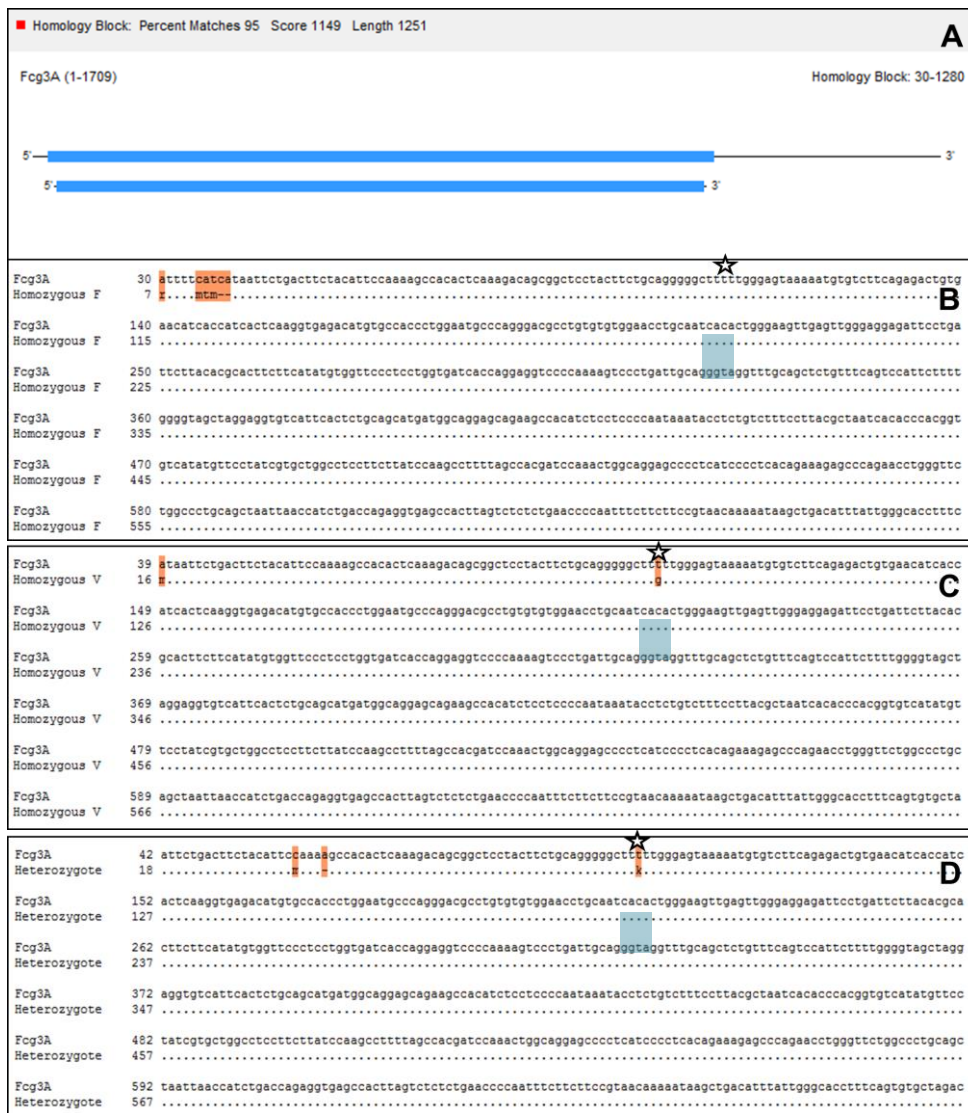


Figure 12: Direct sequencing of the *FCGR3A*-V158F polymorphic region analysed on Sci Ed Central. A) The main region of homology between the known gene sequence (top) and sequenced amplicon (bottom). B), C), D) Homozygous F, homozygous V and heterozygous, respectively comparisons in sequence view showing the *FCGR3A* gene with the sequenced amplicon. Matched bases are represented by a dot; non-matches are highlighted in colour. The location of the SNP is marked with a star and the allele-specific potential restriction site is highlighted in aqua.

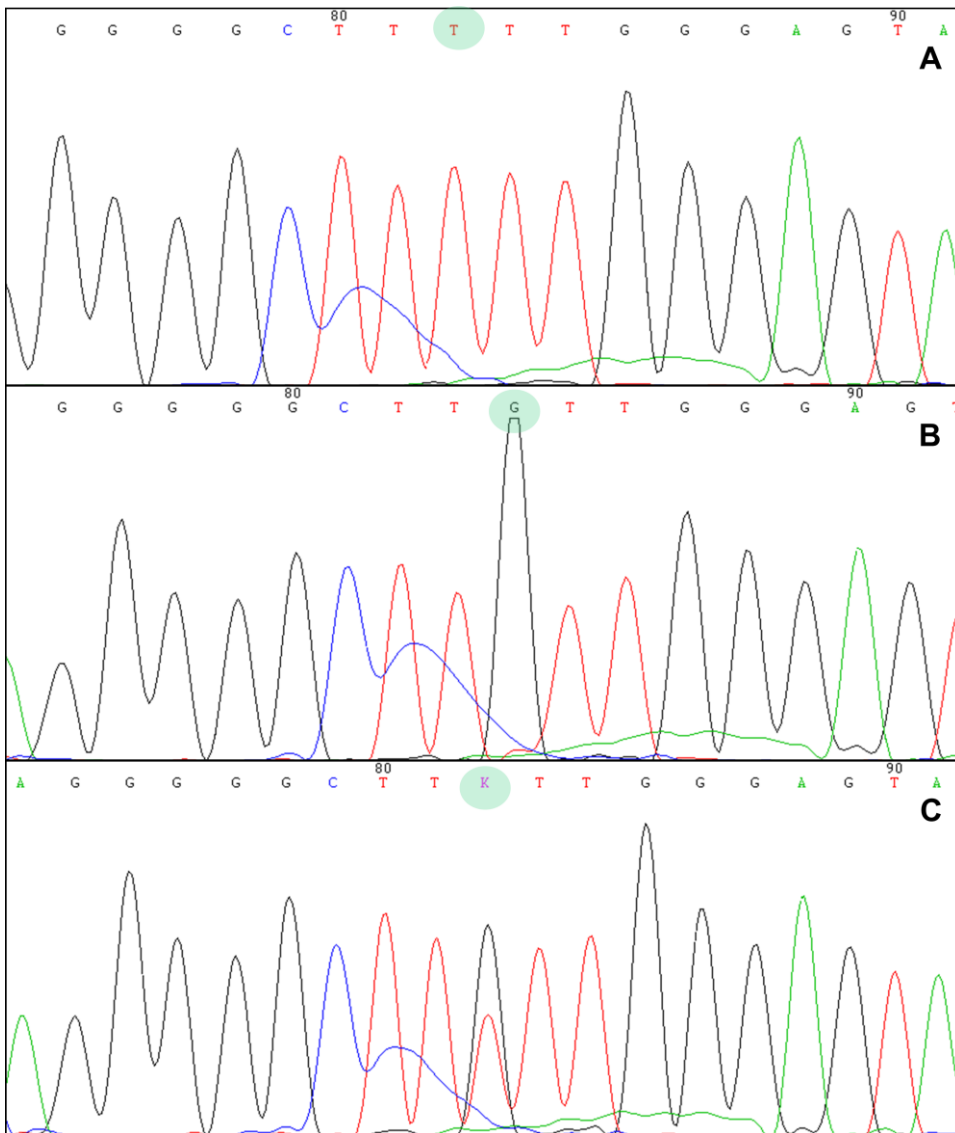


Figure 13: FCGR3A-V158F polymorphism detection: direct sequencing. A), B), C) DNA sequence trace chromatograms of homozygous F, homozygous V and heterozygous amplicons, respectively.

FCGR2A-H131R polymorphism detection

The *FCGR2A*-H131R polymorphism was also genotyped using a published method.²¹⁰ The *FCGR2A* gene sequence, SNP location and primer annealing sites were ascertained (appendix 6). The oligonucleotides had been chosen by Jiang *et al* to specifically amplify the *FCGR2A* gene and not the highly homologous *FCGR2B* and *FCGR2C* genes (forward MP7931: 5'-GGA AAA TCC CAG AAA TTC TCG **C**-3' and reverse MP7932: 5'-CAA CAG CCT GAC TAC CTA TTA **CGC** GGG-3'). The forward primer contains a single-nucleotide substitution (C→G, shown in bold), producing a *Bst*UI restriction site (5'CG⁺CG-3') in FcγR2A-131R encoding DNA but not FcγR2A-131H. The reverse primer contains a double-nucleotide substitution (CT→GC) introducing a *Bst*UI restriction site into all products, regardless of polymorphism, to act as an internal control of enzyme function. The conditions for this single-step PCR assay were 94°C for 3 minutes followed by 35 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 40 seconds, with a final extension of 7 minutes.

Digestion was carried out in 40µl volumes using 14µl PCR product, 2µl *Bst*UI, 4µl buffer R and water, incubated at 37°C for 1½ hours. The 366bp undigested PCR product contained either one or two restriction sites depending on genotype. Post-digestion the identifiable fragment sizes were 343bp for FcγR2A-131H encoding DNA and 322bp for FcγR2A-131R. Following the addition of 10µl DNA load, samples were electrophoresed on high-resolution agarose gels, viewed under UV light and genotype recorded. Different gel concentrations and staining methods were tested; cleanest band separation was achieved using 3% agarose gels containing ethidium bromide (80ml TBE, 2.4g agarose and 5µl ethidium bromide). Normal donor samples were screened to identify homozygous H, homozygous R and heterozygous controls, confirmed by direct sequencing of the amplicon using the reverse primer. Patient samples were analysed in batches of 24 and a negative control containing water instead of DNA was included with each batch. Sample gel photographs and sequencing results are shown in figures 14-16.

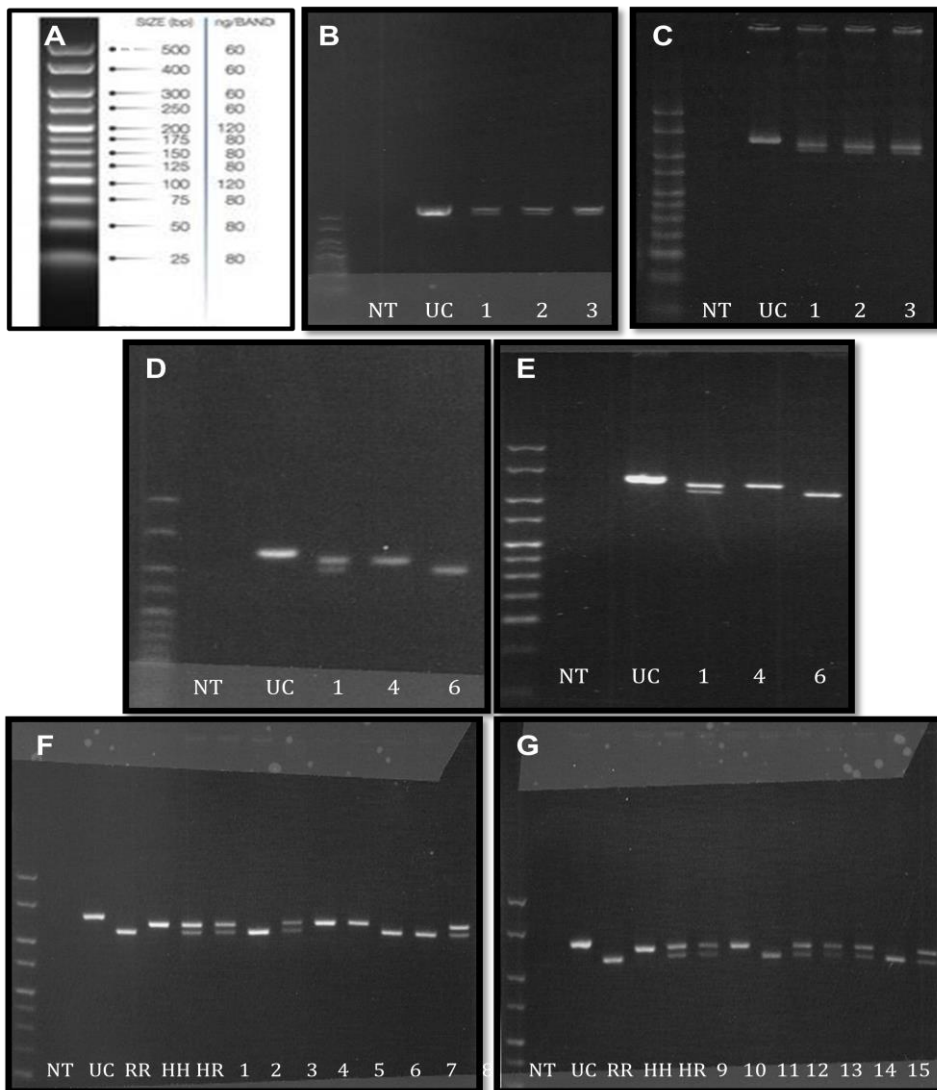


Figure 14: *FCGR2A*-H131R polymorphism determination: high-resolution agarose gel electrophoresis following PCR and *Bst*UI restriction digest. A) Hyperladder V used in first lane of each gel. B-E) Screening samples post digestion run on 1% (B, D) and 3% (C, E) gels stained with gelstar (B, C) and ethidium bromide (D, E). F-G) Patient samples following single-step PCR, restriction digest and electrophoresis on 3% high resolution agarose gels containing ethidium bromide. Patients 4, 5 and 10 are homozygous H; patients 2, 6, 7, 11 and 15 are homozygous R; patients 1, 3, 8, 9, 12, 13, 14 and 16 are heterozygous. NT = no template (blank control), UC = uncut.

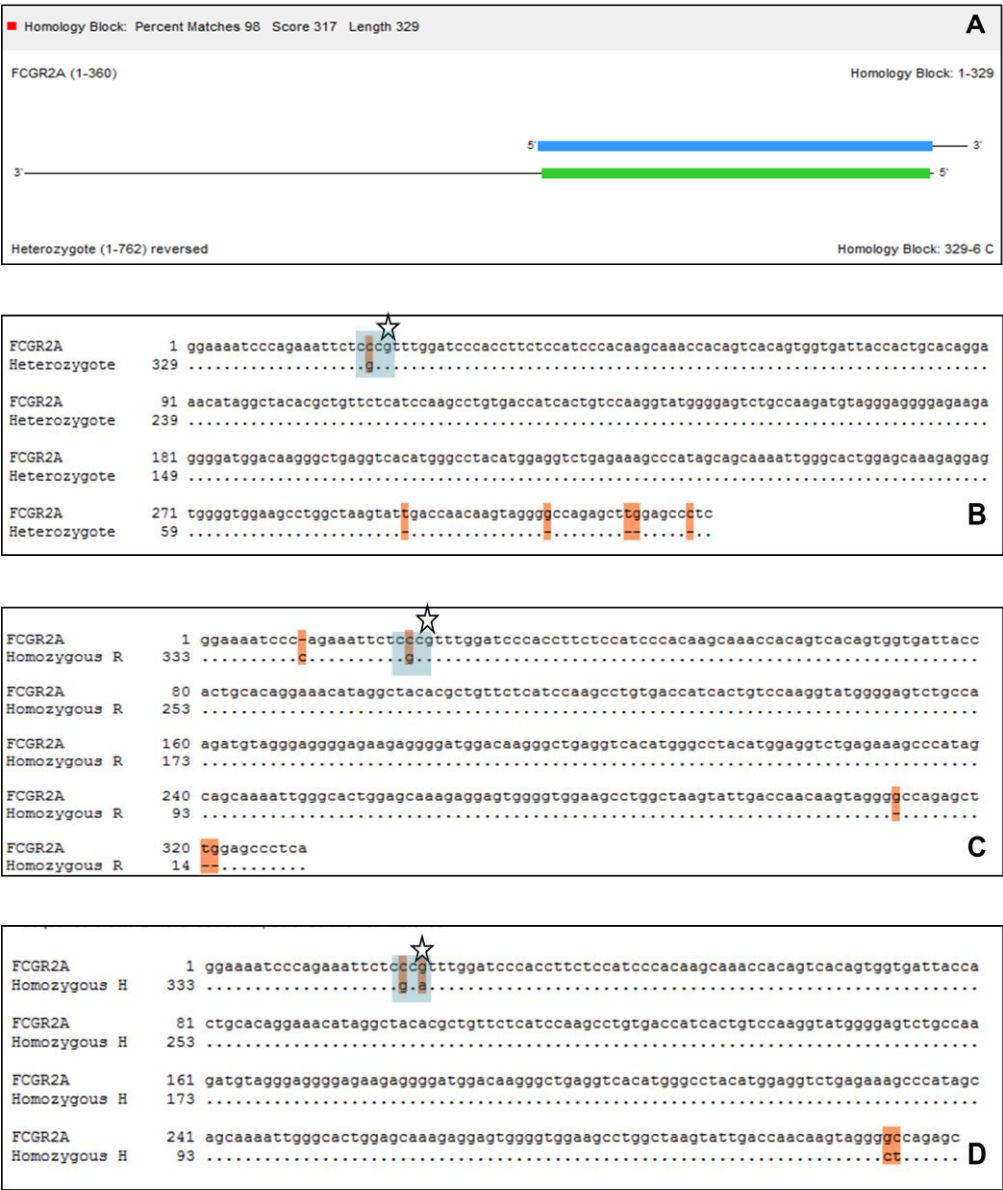


Figure 15: Direct sequencing of the *FCGR2A*-H131R polymorphic region analysed on Sci Ed Central. A) The main region of homology between the known gene sequence (top, blue) and sequenced amplicon (bottom, green). B), C), D) Heterozygous, homozygous R and homozygous H, respectively comparisons in sequence view showing the *FCGR2A* gene with the sequenced amplicon beneath (sequence reversed as reverse primer used). Matched bases are represented by a dot; non-matches are highlighted in colour. The location of the SNP is marked with a star and the allele-specific potential restriction site is highlighted in aqua.

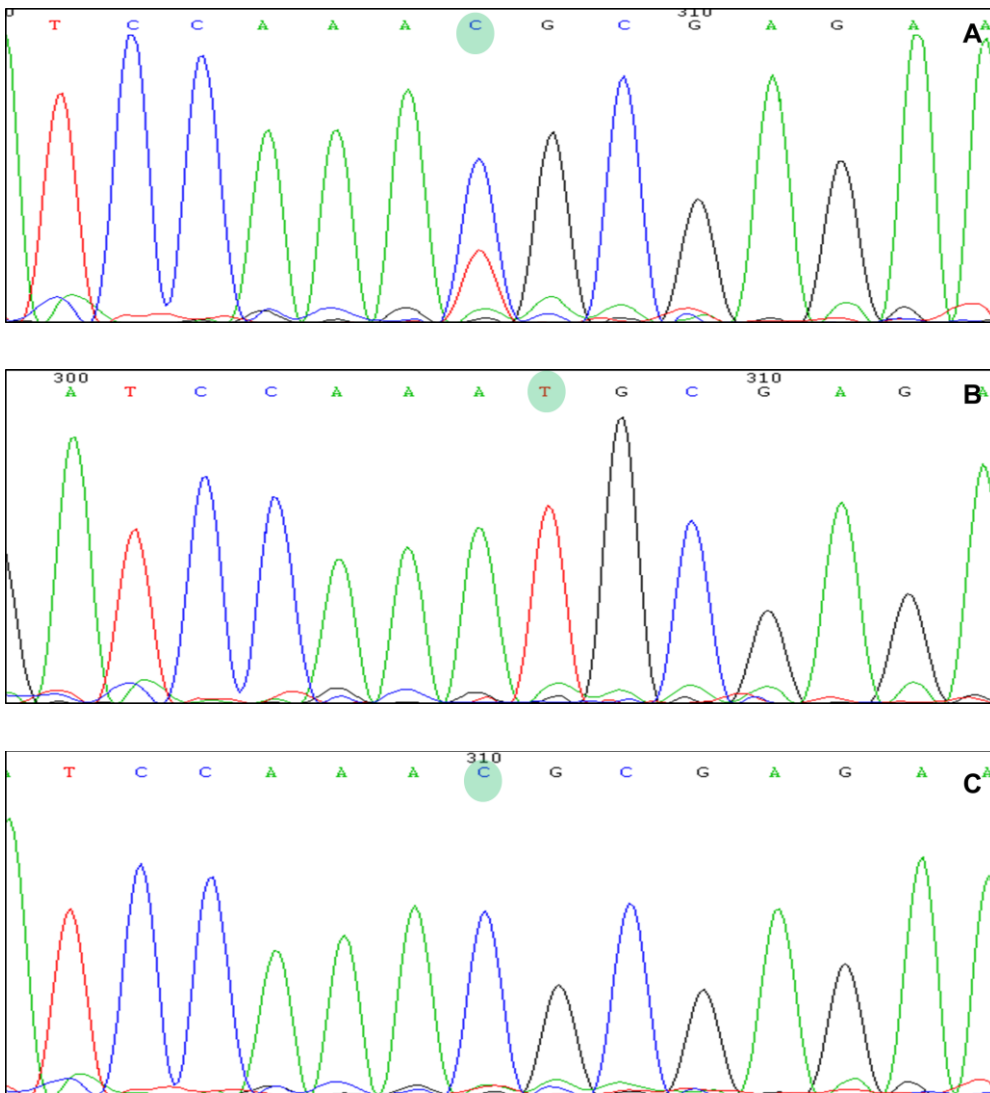


Figure 16: *FCGR2A*-H131R polymorphism detection: direct sequencing. A), B), C) DNA sequence trace chromatograms of heterozygous, homozygous R and homozygous H amplicons, respectively.

***FCGR2B*-I232T polymorphism detection**

The previously published method for detecting the *FCGR2B*-I232T SNP involved a nested PCR with melting point analysis.¹¹⁵ The *FCGR2B* sequence and SNP location were ascertained (appendix 7). A type II restriction endonuclease was identified which was allele-specific; *BsmFI* cuts 10bp down-stream from 5'-GGGAC-3' with a 4bp overhang. New inner primers were designed (by Martin Pule) to amplify a 455bp section of DNA surrounding this site (forward MP8231: 5'-GCT GTG GTC ACT GGG ATT GCT GTA GCG-3' and reverse MP8232: 5'-TAC AAA CCT GAA ATC CGC TTT TTC CTG-3'). This fragment contains one restriction site in the wild-type *FCGR2B*-232I DNA but two sites in *FCGR2B*-232T. A two-step PCR was necessary because the core sequence was repeated at greater than 99% homology. One forward (MP8235: 5'-CGG GTC CTC TGC GGT TTT TTG-3' and two reverse (MP8236: 5'-ACT ACA CTG CTC TCC CCA AGA C-3' and MP8237: 5'-TCC CAC CTG GGC CAG GGC TTG-3') outer primers were designed to anneal at sites of difference from the pseudogene (see appendix 7 for all sites of primer annealing). Outer primers used by Kyogoku *et al*¹¹⁵ were also synthesized (forward MP8233: 5'-AAG GAC AAG CCT CTG GTC AA-3'; reverse MP8234: 5'-CCC AAC TTT GTC AGC CTC AT-3'). Three different pairs of outer primers were tested (figure table + gel picture); bands of the correct size were seen using the previously published primers (A) and the new combination MP8235 with MP8236 (B).

Amplicon	Forward primer	Reverse Primer	Expected size
A ¹¹⁵	MP8233	MP8234	4317bp
B	MP8235	MP8236	1535bp
C	MP8235	MP8237	3145bp

The latter reaction generated a much smaller fragment with no additional bands seen and therefore this amplicon was selected to take forward to the second PCR and digest. The first PCR used the following conditions: initial denaturation for 90 seconds at 96°C followed by 35 cycles of denaturing at 96°C for 30 seconds, primer annealing at 56°C for 30 seconds and extension at 72°C for 2 minutes, followed by a final extension of 5 minutes at 72°C. Conditions for the second PCR were identical except that extension was carried out for 45 seconds per cycle with a final extension

of 4 minutes; the product of the second PCR was 455bp long. The amplicon was purified using QIA quick spin columns and digested with *BsmFI*. Digestion was carried out in 50µl volumes using 2µl enzyme, 5µl buffer 4, 0.5µl BSA, 15µl purified DNA and water, incubated at 65°C for 90 minutes. The FcγR2B-232I allele product contains a single restriction site leading to fragments of 305bp and 150bp. The FcγR2B-232T allele product contains a further restriction site producing fragments of 305bp, 88bp and 62bp. The products were electrophoresed in 3% high-resolution agarose gels stained with ethidium bromide, visualised under UV light and the genotype recorded. Gel photographs and sequencing results are shown in figures 17-19.

TOPO TA cloning

During initial screening for normal controls, no SNP homozygotes (TT) were identified. More samples were screened and direct sequencing used to confirm the amplicon was of the expected sequence. Wild-type homozygotes (II) and heterozygotes (IT) were identified, but no SNP homozygotes (previous studies report the frequency to be 2-3% in Caucasian healthy controls). A SNP homozygote was therefore generated by TOPO-TA cloning of a heterozygote, as described below. The first PCR product from a heterozygote was inserted into a plasmid vector; by chance some vectors then contained FcγR2b-232I encoding DNA and some contained FcγR2b-232T. Bacterial colonies transfected with the vector were cloned and picked individually. This sequence was then extracted from such colonies, providing template for the second PCR which was homozygous in one direction or the other.

The product from the first PCR in a confirmed heterozygote was electrophoresed on 1% agarose gel. The 1.5kb product was identified and cut out of the gel under blue light; gel extraction was then performed using QIA quick spin columns. The product was weighed and 3x QC buffer added before incubation at 50°C for 10 minutes with occasional vortex. An equal volume of isopropanol was added, transferred to the column and centrifuged for 1 minute with the flow-through discarded. Following the addition of 750µl PE buffer, the column was spun for 1 minute and flow-through

discarded. The column was spun for a further minute before transfer to a clean 1.5ml eppendorf tube and elution with 30µl nuclease-free water.

A TOPO-TA cloning kit (Invitrogen) with pCR[®]II-TOPO[®] plasmid vector (figure 20) was used. Topoisomerase I binds duplex DNA and cleaves the phosphodiester backbone after 5'-CCCTT in one strand of the vector. Single 3'-thymidine overhangs allow efficient ligation with the single deoxyadenosine added to the ends of PCR products by *Taq* polymerase (nontemplate-dependent terminal transferase activity). The vector contains antibiotic resistance genes and the cloning site is flanked by *Eco*RI restriction sites (G⁺AATTC) to allow extraction of the cloned sequence. A 50µl aliquot of high-efficiency competent *E. coli* bacteria (New Eng Biolabs C2987H) was defrosted on ice. A combination of 1µl TOPO-kit salt mix, 1µl vector and 2µl water were gently mixed with 1µl cleaned PCR product and incubated at room temperature for 15 minutes. In a 0.5ml eppendorf tube, 2µl of this ligation reaction was added to 25µl cells and kept on ice for 45 minutes. The bacteria were transfected by 35 second heat shock at 42°C in a water bath, then returned to ice for 2 minutes. The bacteria were added to 250µl SOC medium in a 12ml round bottom falcon tube and incubated on a bacteria shaker at 200rpm at 37°C for one hour. An agar plate containing carbenicillin was dried and evenly spread with 50µl X-Gal. The plate was inoculated with 250µl SOC/bacteria using the Pasteur method with a disposable loop spreader, and incubated at 37°C overnight. The colonies were picked the next day (only untransformed bacteria express β-galactosidase and cleave X-Gal; blue/white screening used), added to 4ml LB medium with carbenicillin and kept on a bacterial shaker at 200rpm at 37°C overnight.

The next day, 1.5ml of each sample was transferred to an eppendorf tube, spun for 10 minutes at 14000rpm and the supernatant discarded. The plasmid was then extracted using commercial mini-prep kits (Qiagen). The pellet was resuspended in 250µl P1 buffer containing EDTA. Cell lysis was achieved by adding 250 µl alkaline P2 buffer and shaking well to mix. After 5 minutes 350µl neutralisation buffer N3 was added and the sample mixed gently but thoroughly. The sample was centrifuged for 10 minutes and the supernatant applied to a Qiaprep spin column. The column was spun for 1 minute at 1300rpm, the flow-through was discarded and 750µl PE wash buffer was added. This was spun for 1 minute, flow-through

discarded and spun for a further minute. The column was then placed in a clean 1.5ml eppendorf tube and the DNA eluted into 50µl water.

The *FCGR2B* DNA sequence was then extracted from the plasmid by *EcoRI* digest. The digestion reaction containing 1.5µl enzyme, 3µl buffer 3, 3µl DNA sample and 22.5µl water was incubated at 37°C for 2 hours. The digested product was run on 1% agarose gels containing ethidium, with 10µl sample and 2µl DNA load per well. Those with identifiable bands of the correct size were sent for sequencing and as expected some had a T and some a C at the polymorphic site of interest. These were then used as template for the second PCR reaction and subsequent *BsmFI* digestion and gel electrophoreses. Bands of the predicted sizes were found and these cloned PCR products were subsequently used as homozygous normal controls during patient sample processing.

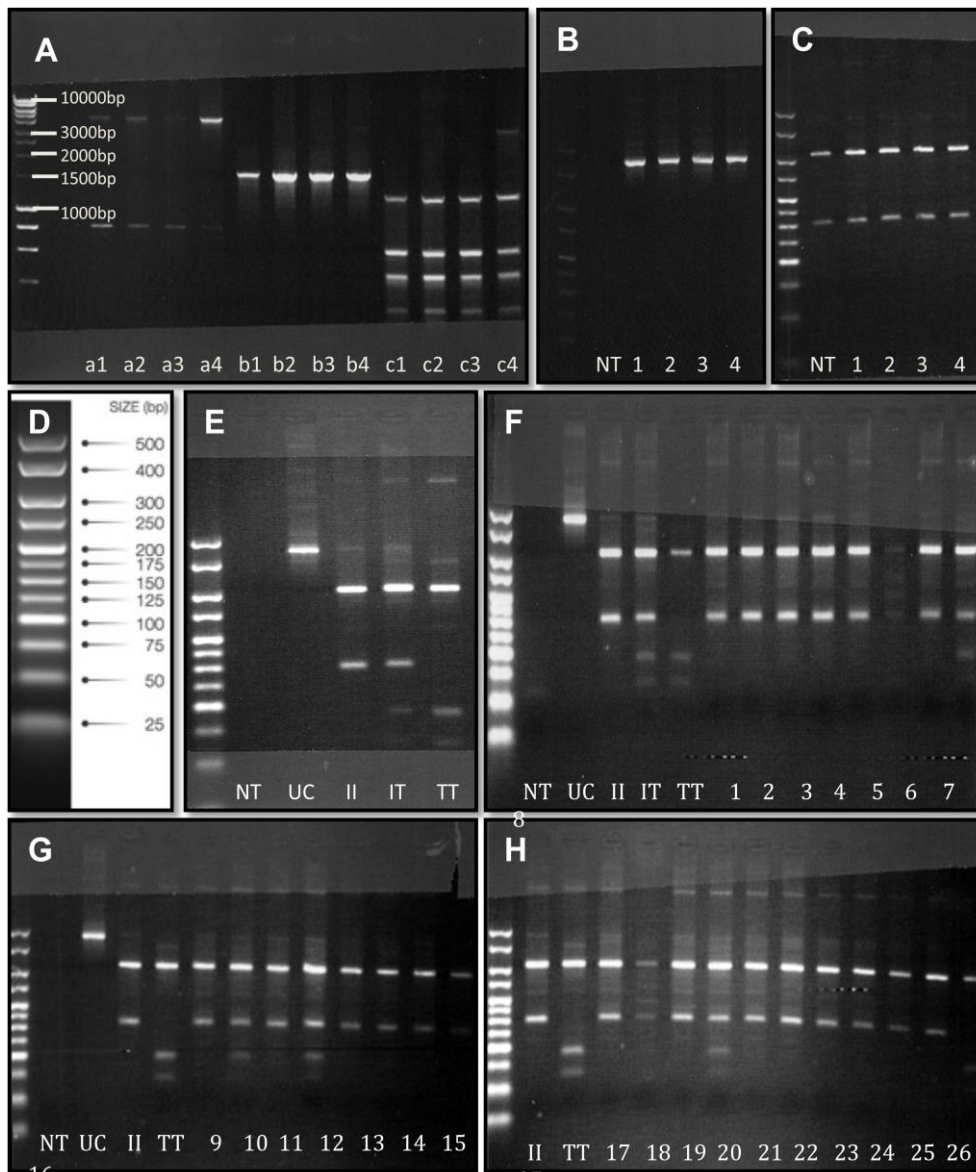
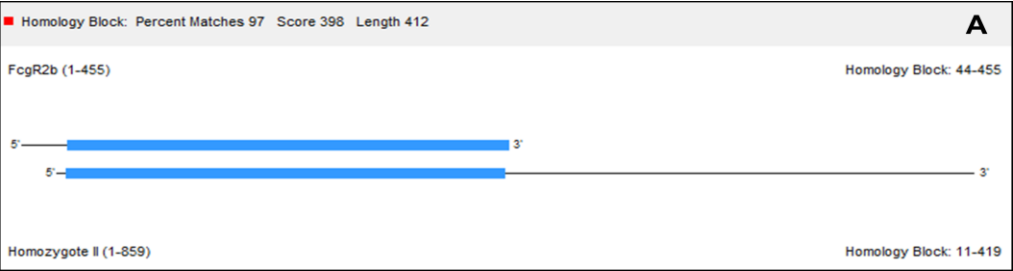


Figure 17: *FCGR2B*-I232T polymorphism determination: high resolution agarose gel electrophoresis following nested PCR and *Bsm*FI restriction digest, photographed under UV light. A) Three different outer primer pairs were tested using four screening samples, with expected amplicon sizes a) 4317bp, b) 1535bp and c) 3145bp. Primer pair b) gave a band of the expected size with no other bands so this amplicon was then used in the second (nested) PCR. B) Undigested nested PCR product from screening samples corresponding to the expected band size of 455bp. C) Digested nested PCR product from screening samples corresponding with expected I homozygous band sizes of 305bp and

Patients, Materials and Methods

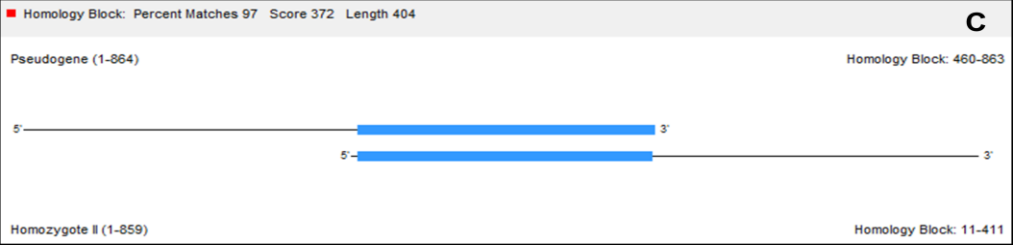
150bp. D) Hyperladder V used in figures B)-H). E) Controls used with each patient batch. The negative control contained no template (NT); uncut (UC) product acted as an internal control of enzymatic activity. The T homozygote has three bands of 305bp, 88bp and 62bp; control product was generated by TOPO-TA cloning as no screening sample was identified. Heterozygotes have four bands of 305bp, 150bp, 88bp and 62bp. F), G), H) Patient samples. Patients 8, 10, 12 and 20 are heterozygous; patient 27 is homozygous T; patient 6 has an unclear result repeated in the next batch; the remaining patients are homozygous I.

Patients, Materials and Methods



FcgR2b	44	ttgggtccacccttccactgccc	tgagggtcaagggagcccttccctctgttctcctgctcacaatgtac	ctttattagttgg
Homozygote I	11
FcgR2b	134	tggagaaacctcggttaagcaggaggcataagtcacagccacagaaacctgtgcagatgaggctggggatgtagtgctgcagaagtgc		
Homozygote I	98
FcgR2b	224	agtgactcagacacagaagagcttcgggtgacaagcactaggacatagcattggatggggggagggtgggacaaggagagtactgcctgt		
Homozygote I	188
FcgR2b	314	cctgatgtctgtcttccctagctcccagctcttcaccgatggggatcattgtggctgtggtcactgggat	☆	ctgtagcggccattgttg
Homozygote I	278
FcgR2b	404	ctgctgtagtgcccttgatctactgcaggaagcggatttcagg		tttgta
Homozygote I	368

B



Pseudogene	460	ttgggtccacccttccactgccc	tgagggtcaagggagcccttccctctgttctcctgctcacaatgtac	ctttattagttgg
Homozygote I	11
Pseudogene	550	tggagaaacctcggttagcaggaggcataagtcacagccacagaaacctgtgcagatgaggctggggatgtagtgctgcagaagtgc		
Homozygote I	98
Pseudogene	640	agtgactcagacacagaagagcttcgggtgacaagcactaggacatagcattggatggggggagggtgggacaaggagagtactgcctgt		
Homozygote I	188
Pseudogene	730	cctgatgtctgtcttccctagctcccagctcttcaccgatggggatcattgtggctgtggtcactgggat	☆	ctgtagcggccattgttg
Homozygote I	278
Pseudogene	820	ctgctgtagtgcccttgatctactgcaggaagcggatttcagg		tttgta
Homozygote I	368

D

FcgR2b	35	tcgaggtcttgggcccacccttccactgcccctgagggctcaagggagcccttccctctgttctcctgctcacaatgtac	ctttattagttgg
Heterozygote	10
FcgR2b	125	ttagtttggtggagaaacctcggttaagcaggaggcataagtcacagccacagaaacctgtgcagatgaggctggggatgtagtgctgc	
Heterozygote	96
FcgR2b	215	gcagaagtgagtactcagacacagaagagcttcgggtgacaagcactaggacatagcattggatggggggagggtgggacaaggagagt	
Heterozygote	186
FcgR2b	305	actgctgtcctgatgtctgtcttccctagctcccagctcttcaccgatggggatcattgtggctgtggtcactgggat	☆
Heterozygote	276
FcgR2b	395	ccattgtgtctgtagtgcccttgatctactgcaggaagcggatttcagg	tttgta
Heterozygote	366

E

FcgR2b	1	gctgtgctccatagagtaatgatgctccagctatgagaggtcttggggccacccttcccactgcccctgagggctaaggggagcccttc
Homozygote I	916
FcgR2b	91	cctctgttccctgctcacaatgtacctttattagtttgggtggagaaacctcggttaagcaggaggcataagtcacccacagaaacc
Homozygote I	826
FcgR2b	181	ctgtgcagatgaggtctggggatgtagtgagtgctgcagagtgagtgactcagacacagagagcttcgggtgacaagcactaggacata
Homozygote I	736
FcgR2b	271	gcattggatgggggggagggtgggacaaggagagtactgctgtcctgatgtctgtcttccctagctcccagctcttcaccgatggggatc
Homozygote I	646
FcgR2b	361	attgtggctgtgtgtcactggga [*] tgctgtagcggccattgttgcctgctgtagtgcccttgatctactgcaggaagcggatttcaggt
Homozygote I	556
FcgR2b	451	ttgta
Homozygote I	466

F

Figure 18: Direct sequencing of the *FCGR2B*-I232T polymorphic region analysed on Sci Ed Central. A), C) The main region of homology between the sequenced homozygous I amplicon (bottom in each picture) and known gene sequence and pseudogene, respectively. B), D) Comparisons in sequence view showing the *FCGR2B* gene (B) or pseudogene (D) with the sequenced homozygous I amplicon beneath (sequence reversed as reverse primer used). Matched bases are represented by a dot; non-matches are highlighted in colour. The location of the SNP is marked with a star. Comparisons showing the gene sequence compared with heterozygous and homozygous T amplicons are shown in E) and F), respectively.

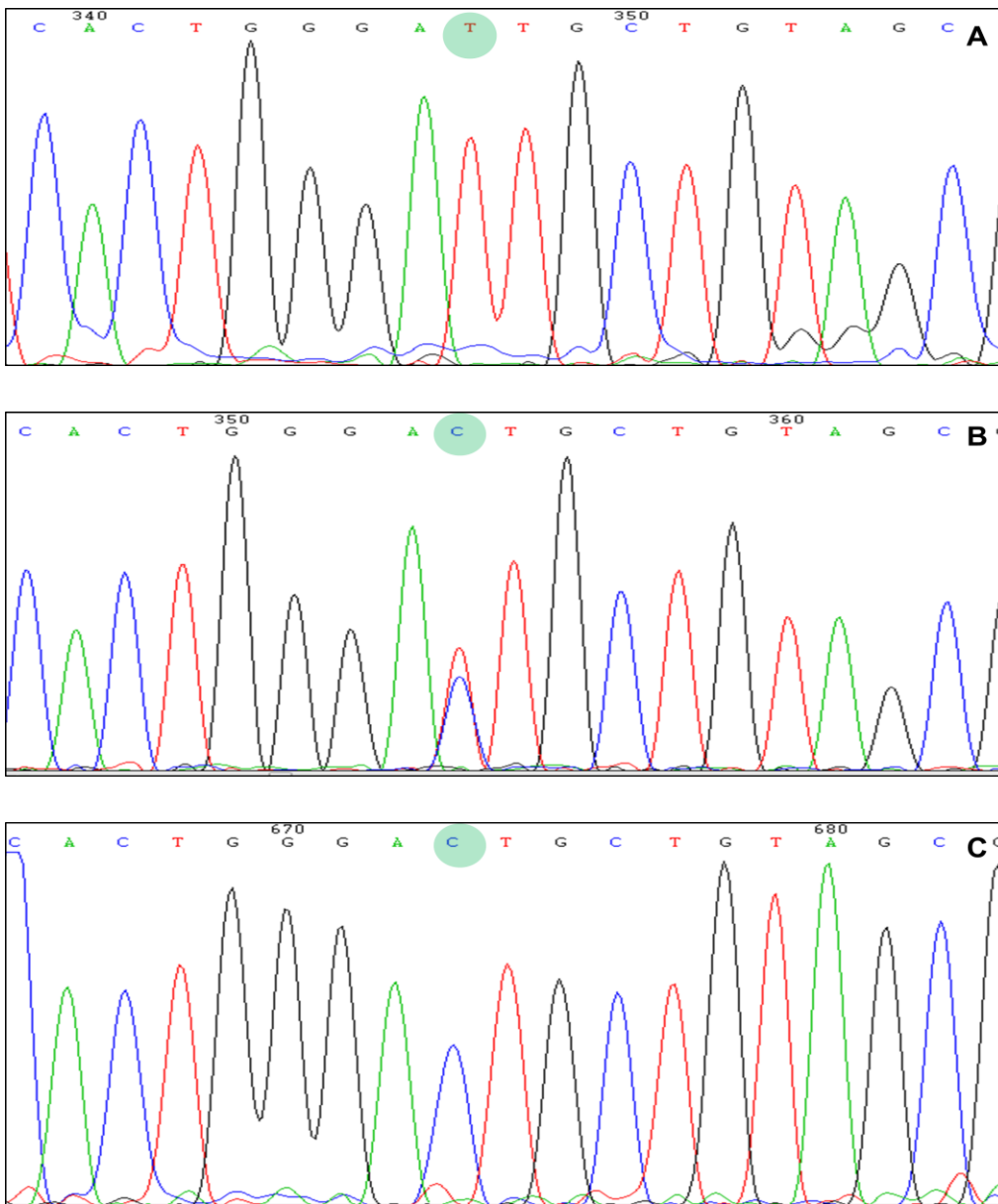


Figure 19: *FCGR2B*-I232T polymorphism detection: direct sequencing. DNA sequence trace chromatograms of homozygous I (A), heterozygous (B) and homozygous T (C) amplicons. The location of the polymorphism is highlighted in green.

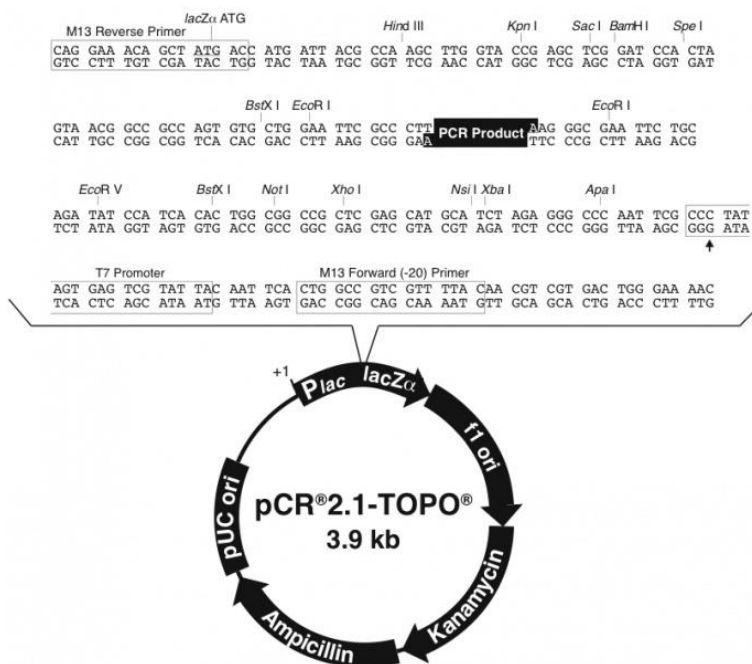


Figure 20: Schematic representation of the pCR®II-TOPO® plasmid vector showing the cloning site flanked by EcoR I digestion sites. *LacZα* encodes β-galactosidase which will produce a blue dye from the substrate X-gal. Successful insertion of the amplicon disrupts this gene and prevents β-galactosidase expression; colonies of transformed bacteria will be white. The plasmid also contains antibiotic resistance genes.

C1QA-Gly70^{GGG/GGA} polymorphism detection

The C1QA gene sequence and location of the SNP of interest were ascertained (appendix 8). Initially, published primers were used in a single-step PCR but the correct amplicon was not generated. Collaboration with investigators in Montpellier was initiated and it was decided to follow the same methods as much as possible, with a joint publication in mind. The primers were therefore ordered to match those used by the Montpellier group (forward MP11356: 5'-GCC TTA AAG GAG ACC AGG GGG AAC-3' and reverse MP11357: 5'-CCC TTG AGG AGG AGA CGA TGG AC-3'). PCR conditions used by the Montpellier group were tried but no bands were visible when the product was electrophoresed. Following optimisation of the conditions for our laboratory the PCR produced detectable product and thus the

following conditions were used for this single-step PCR assay: initial denaturation at 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 45 seconds, with a final extension of 5 minutes.

Digestion was carried out in 50µl volumes using 12µl PCR product, 2µl *Apa*I, 5µl buffer 4, 0.5µl BSA and water, incubated at 37°C for 2 hours. The 348bp undigested PCR product contained either two or three restriction sites (GGGCC⁺C) depending on genotype. Post-digestion the identifiable fragment sizes were 281bp for C1qa-Gly70_{GGA} encoding DNA and 262bp for C1qa-Gly70_{GGG}. Following the addition of 10µl DNA load, samples were electrophoresed on high resolution agarose gels, viewed under UV light and genotype recorded. Different gel concentrations and staining methods were tested; cleanest band separation was achieved using 3.5% agarose gels containing ethidium bromide (80ml TBE, 2.4g agarose and 5µl ethidium bromide). Normal donor samples were screened to identify homozygous G, homozygous A and heterozygous controls, confirmed by direct sequencing of the amplicon using the reverse primer. Patient samples were analysed in batches and a negative control containing water instead of DNA was included with each batch. Gel photographs and sequencing results are shown in figures 21-23.

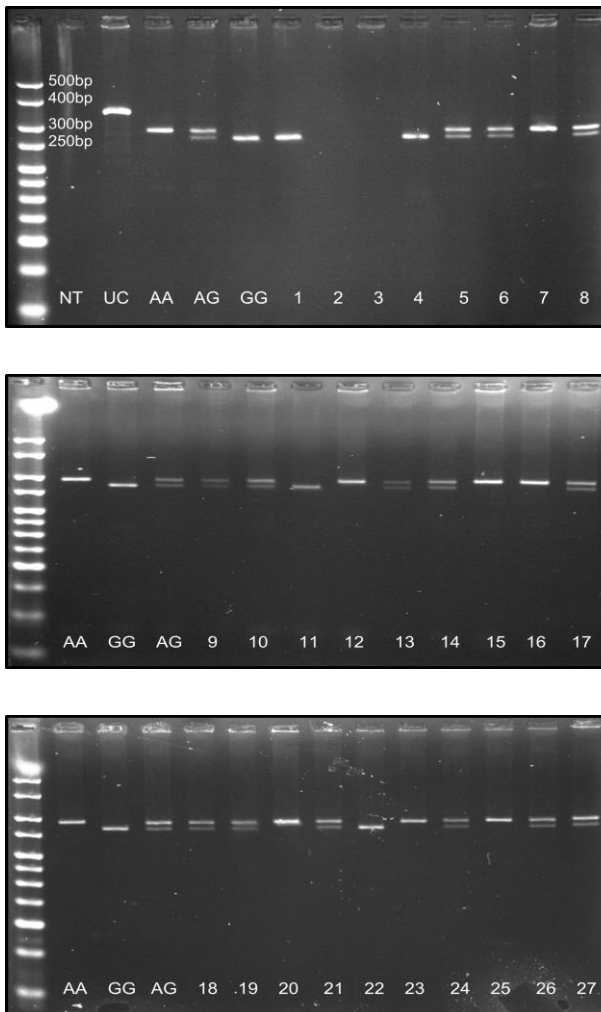


Figure 21: C1QA-Gly70 polymorphism detection. The PCR product was digested with *Apal* restriction enzyme, had DNA loading dye added and was electrophoresed on 3.5% agarose gels containing ethidium bromide, then viewed under UV light. The first lane in each gel contains hyperladder V. Each gel has three positive controls: in A homozygotes a single 281bp band is seen, G homozygotes have a single 262bp band and heterozygotes have both bands. The uncut (UC) amplicon is included as a control of enzymatic activity (348bp); the blank control used no template (NT). Patients 7, 12, 15, 16, 20, 23 and 25 are A homozygotes; patients 1, 4, 11 and 22 are G homozygotes; patients 5, 6, 8, 9, 10, 13, 14, 17, 18, 19, 21, 24, 26 and 27 are heterozygotes; no product was obtained from patients 2 and 3 and these were repeated in a subsequent batch.

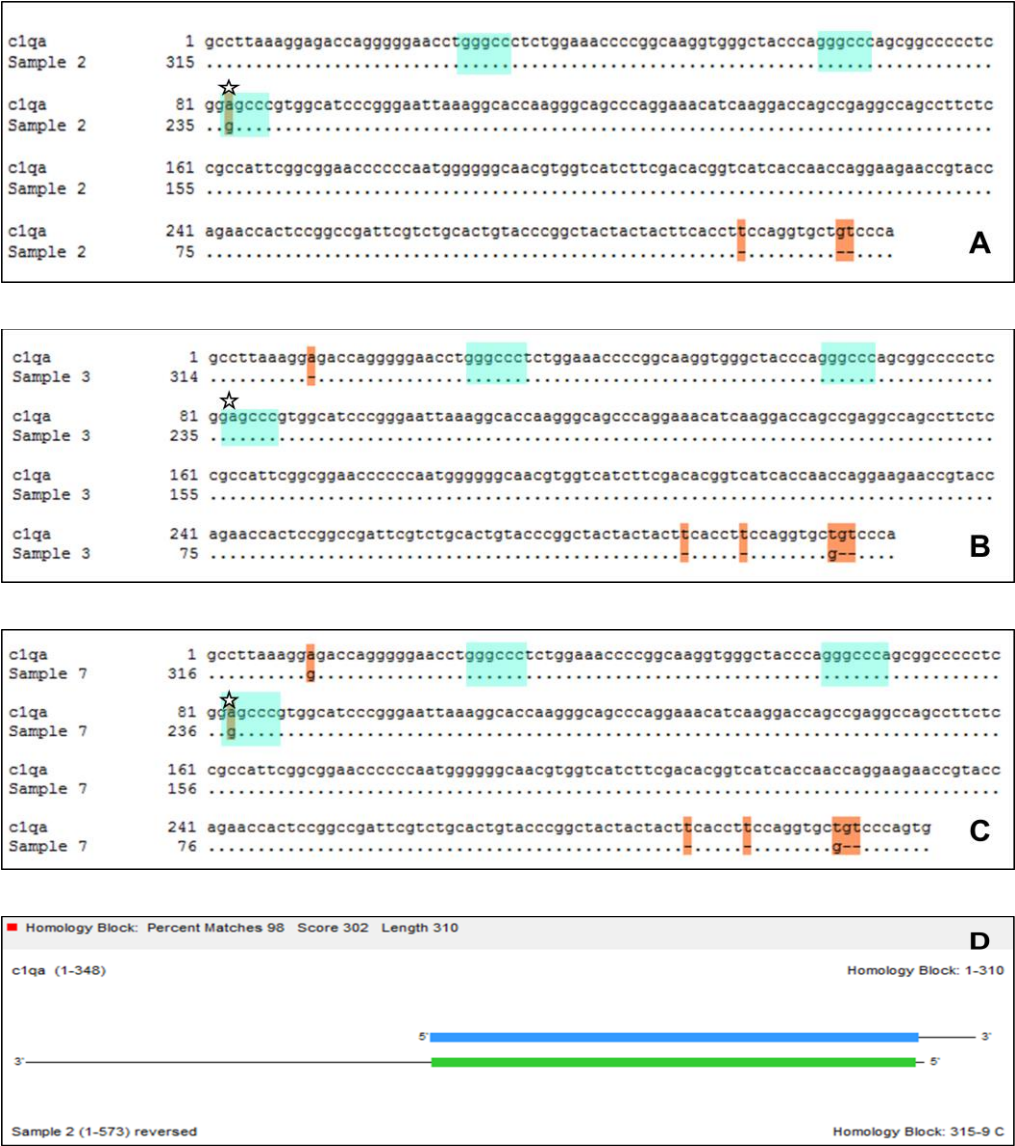


Figure 22: C1QA-Gly70 polymorphism detection: direct sequencing. Using Sci Ed Central the known C1QA gene sequence is compared with screening samples 2 (fig A; heterozygote), 3 (fig B; homozygous A) and 7 (fig C; homozygous G). The C1QA sequence is shown on top in each picture, with the sequenced amplicon beneath (sequence reversed as reverse primer used for sequencing). Matched bases are represented by a dot; non-matches are highlighted in colour. Potential *Apal* restriction sites (GGGCC⁺C) are highlighted in aqua; the SNP location is marked with a star. Fig D shows the main region of homology between the known C1QA gene sequence (above; highlighted blue) and sequenced amplicon (below; highlighted green).

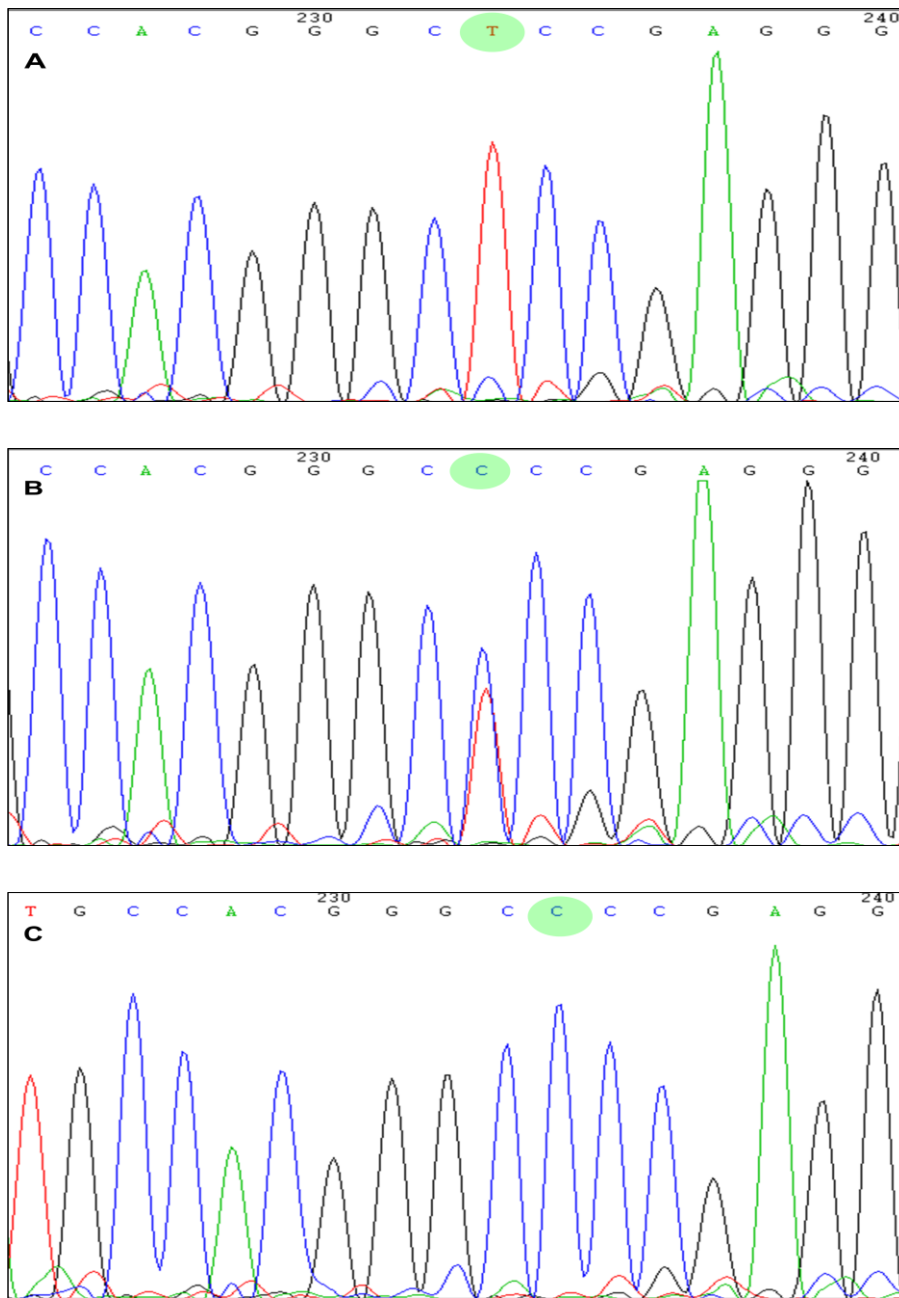


Figure 23: C1QA-Gly70 polymorphism detection: direct sequencing. DNA sequence trace chromatograms of homozygous A (A), heterozygous (B) and homozygous G (C) amplicons. The location of the polymorphism is highlighted in green.

C3-R102G polymorphism detection

The C3 gene sequence and location of the SNP were ascertained (appendix 9). Primers described by the Montpellier group were made (forward MP11350: 5'-CCA AAA CGG CCA CCT CGG AA-3' and reverse MP11351: 5'-CCG TCC GGC CCA CGG GTA GC-3') and used to amplify a 431bp segment of DNA, containing the polymorphic site. The conditions for the single-step PCR were initial denaturation at 94°C for 5 minutes followed by 30 cycles of 94°C for 1 minute, 65°C for 30 seconds and 72°C for 30 seconds, with a 5 minute final extension at 72°C. Again, these conditions were slightly different than those employed by the Montpellier group.

The PCR product was purified using QIA quick spin columns as before. Digestion was carried out in 20µl volumes using 6µl amplicon, 0.5µl *Hha*I, 2µl buffer 4, 0.2µl BSA and water, incubated at 37°C for 2 hours. A single GCG⁺C restriction site was present in C3-102-R encoding DNA, yielding fragments of 264bp and 167bp. C3-102-G encoding DNA remained uncut. Following the addition of 5µl DNA loading dye, samples were electrophoresed on agarose gels, viewed under UV light and the genotype recorded. Different gel concentrations and staining methods were tested; adequate band separation was achieved using 2% gels (using 90ml TBE, 1.8g fine resolution agarose and 5µl ethidium bromide), Normal donor samples were screened to identify homozygous G, homozygous R and heterozygous controls, confirmed by direct sequencing of the amplicon using the reverse primer. Patient samples were analysed in batches of 27 and a blank control run in parallel with each batch. Gel photographs and sequencing results are shown in figures 24-26.

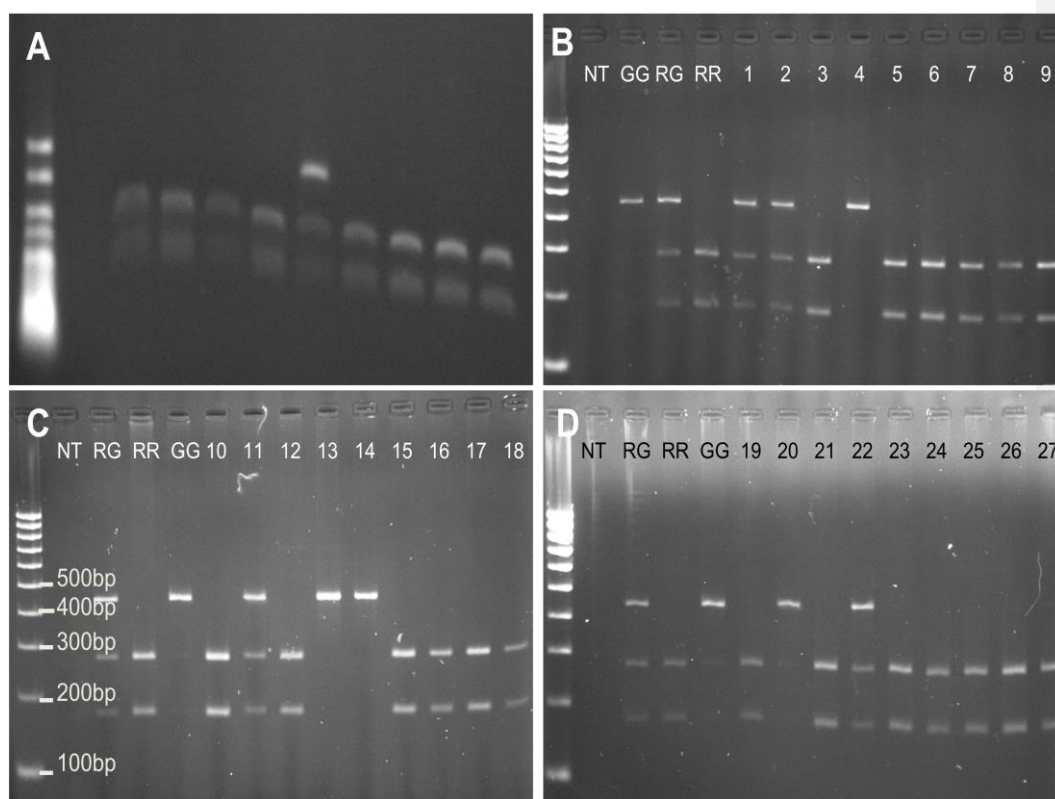


Figure 24: C3-R102G polymorphism analysis: PCR and *HhaI* restriction digest followed by agarose gel electrophoresis, photographed under UV light. The expected band sizes are 264bp and 167bp for the C3-102-R allele containing the restriction site and 431bp for the C3-102-G allele which lacks the restriction site. Three bands are seen for heterozygotes. A) Screening samples run on 1% agarose gel containing ethidium bromide, with hyperladder V in lane 1. Sample 5 appears to be heterozygous with the others all homozygous R. B), C), D) Patient samples run on 2% fine grade agarose gels containing ethidium bromide, with hyperladder IV in lane 1. Each gel has a negative control (NT = no template) and three positive controls. Patients 3, 5-10, 12, 15-19, 21 and 23-27 are homozygous R; patients 4, 13, 14 and 20 are homozygous G; patients 1, 2, 11 and 22 are heterozygous.

Patients, Materials and Methods

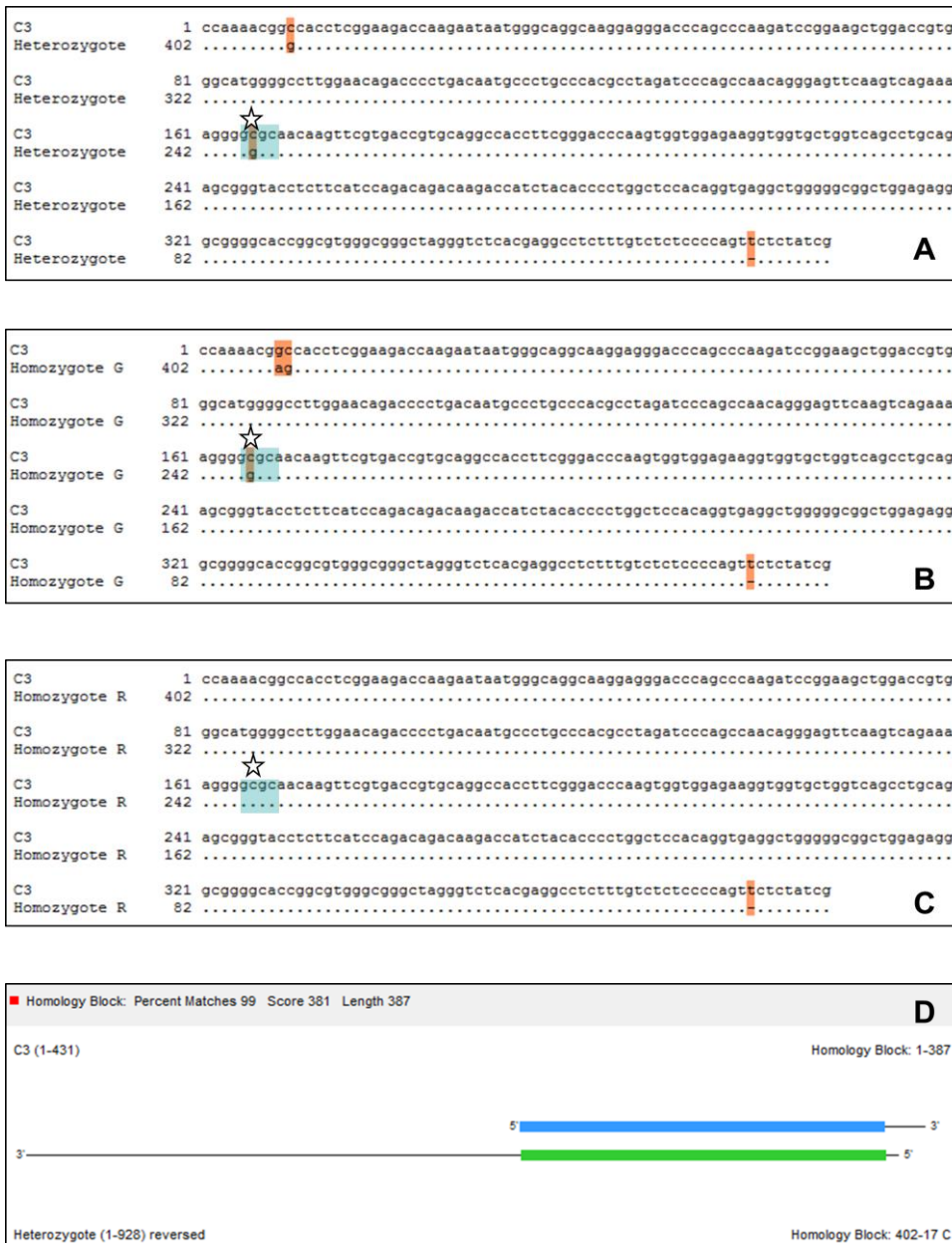


Figure 25: C3-R102G polymorphism analysis: direct sequencing. Using Sci Ed Central the known C3 gene sequence is compared with screening samples found by restriction digest and gel electrophoresis to be A) heterozygous, B) homozygous G, and C) homozygous R. The C3 sequence is shown on top in each picture with the sequenced amplicon beneath (reversed as reverse primer used). Matched bases are represented by a dot; non-matches are high-lighted in orange. The potential *Hha*I restriction site (GCG⁺C) is high-lighted in aqua and a star marks the location of the SNP. Fig D shows the region of homology between the known C3 gene and the sequenced amplicon.

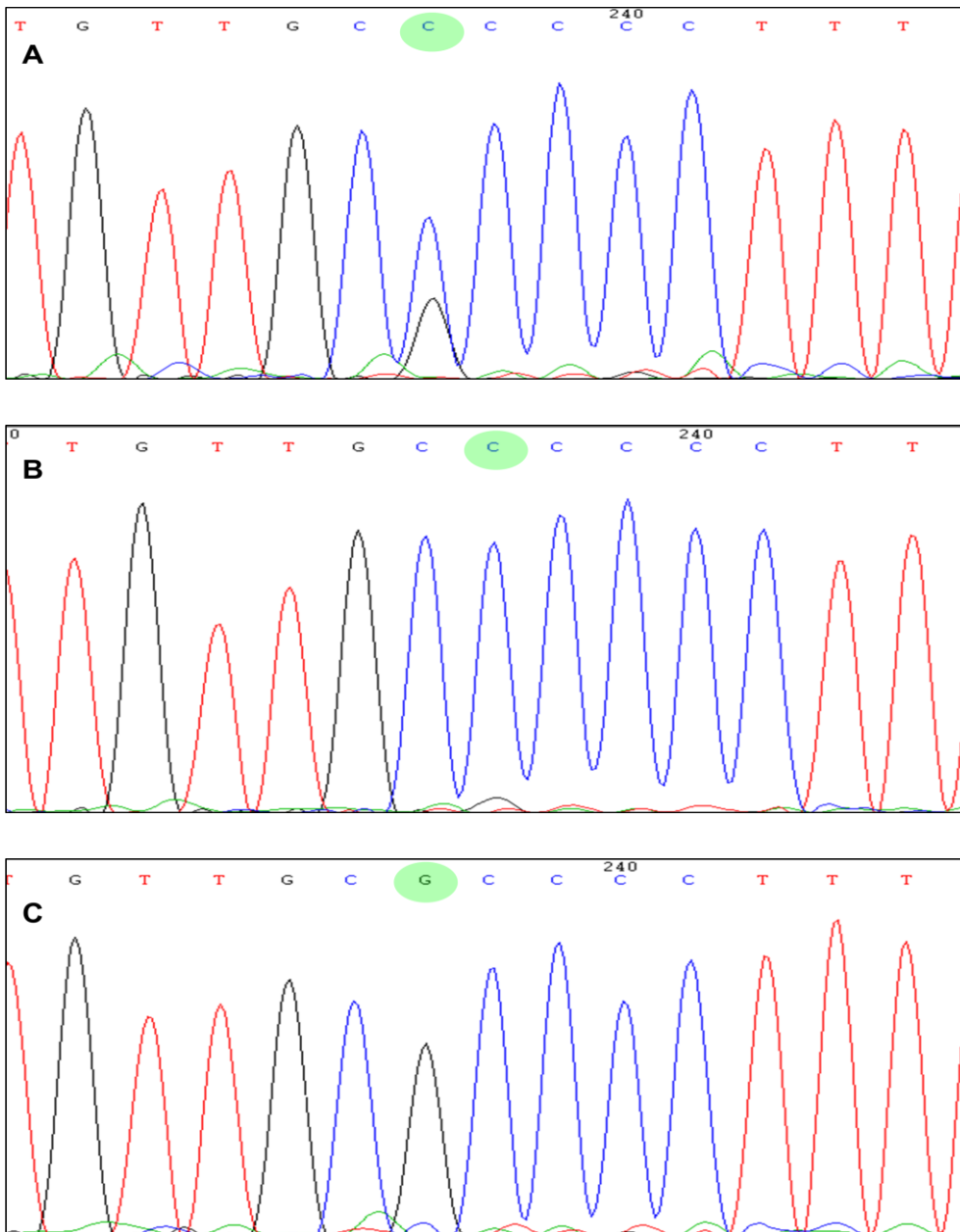


Figure 26: C3-R102G polymorphism analysis: direct sequencing. DNA sequence trace chromatograms of heterozygous (A), homozygous R (B) and homozygous G (C) amplicons. The location of the polymorphism is highlighted in green.

Collaborations and future directions

Constitutional DNA is now stored from the majority of patients entered on this trial, and the clinical dataset has been thoroughly reviewed and cleaned up such that the outcome assessments are robust and reliable. The trial has completed accrual but will continue to collect data for years to come, in order to investigate long-term survival differences between randomisation arms. In the future it will be possible to look for any OS differences by polymorphism status. Meanwhile, the clinical data linked with patient DNA is a valuable resource for investigation of other putative patient factors in FL natural history and response to rituximab therapy. Ideally, tumour DNA would also be available and linked to this to allow evaluation of biomarkers.

Patient DNA at approximate 250 ng/μl dilution were sent in mini-racked tubes (Micronic) containing 10μl sample each, to collaborating groups in Montpellier, France (Cartron *et al*) and St Barts, London (Fitzgibbon *et al*). Two polymorphisms in the *ITGAM* gene encoding complement c3 receptor (CR3/CD11b) were investigated in the laboratory of the Montpellier group. The polymorphism of interest theorised to modify the C3/CD11b interaction *ITGAM*-M425T, and a control polymorphism *ITGAM*-P1130S localised outside the C3/CD11b interaction site, were previously thought to influence PFS after rituximab therapy in FL.⁹¹ The St Barts group analysed patient samples for the rs2072407 mutation in the *EZH2* gene which encodes a histone methyltransferase. Mutations within this gene have been reported in several malignancies, including lymphoma.²¹¹ Ultimately, a comparison between constitutional and tumour DNA with link to clinical outcome should address whether this mutation has significant influence on response to rituximab therapy.

In order to establish a tumour DNA archive, diagnostic tumour blocks will be recalled from participating sites. In preparation for this, whole genome amplification from formalin-fixed, paraffin-embedded (FFPE) tissue has been performed using the REPLI-g kit (Qiagen). The process prepares and randomly ligates fragmented DNA (damaged in the fixing process) before amplification.²¹² This kit has been tested on spare tonsil specimens, and subsequent PCR for the FCGR3A-V158F polymorphism yielded a result in two of the three cases.

Results

Availability of genetic material

Of 276 patients randomised to receive rituximab on trial, availability of genetic material allowed *FCGR3A*-V158F polymorphism status to be ascertained in 259. In 234, genomic DNA extracted from peripheral blood or bone marrow aspirate was used, 2 patients had genomic DNA recovered from stored glass slides (one bone marrow aspirate and one lymph node biopsy), and 23 patients with no available genomic DNA had cDNA generated from RNA. No sample was received from 14 patients (8 of those from outside the UK) and 3 samples were of poor quality and yielded no results.

Some of the smaller and poorer quality samples became exhausted and as such fewer results were obtained for the other polymorphisms: 256 for *FCGR2A*-H131R and *FCGR2B*-I232T, 250 for *C1QA*-Gly70_{GGG/GGA} and 242 for *C3*-R102G.

Missing samples for the 187 patients randomised to watch and wait (arm A) were not pursued so aggressively; availability of genetic material allowed *FCGR3A*-V158F polymorphism status to be ascertained in 87. These results are not included in the following analyses, which relate purely to patients randomised to receive rituximab. The results from these samples are shown in Appendix X.

***FCGR3A*-V158F**

Observed polymorphism frequencies were as follows: 111 FF(43%); 117 VF(45%); 31 VV(12%). The groups were broadly comparable in terms of age, gender, stage, marrow involvement and FLIPI score (table 14). The VV group were slightly younger (median age VV, 55 years; VF, 60 years; FF, 59 years), and a greater proportion had low-risk FLIPI scores (0/1 VV 48%; VF 28%; FF 40%) and excellent performance status (PS0 VV 94%; VF 92%; FF 88%). The only statistically significant difference in the populations was in the proportion of patients in the rituximab maintenance randomisation arm. 72% of VF/FF patients were randomised to R4+M; however for

FCGR3A-158V homozygotes only 52% were in this arm, with 48% randomised to R4 only ($p=0.04$).

With a median follow-up of 46 months, there was no significant difference in overall response rate (ORR) or complete response rate (CRR) by polymorphism at 7, 13 or 25 months (table 5). This was true comparing heterozygotes separately or combined with either group of homozygotes (table 6). The ORR (CRR) were FF:87%(63%); VF:85%(55%); VV:76%(41%) at month 7 and FF:73%(65%); VF:71%(61%); VV:72%(61%) at month 25. Contrary to previous reports, there was a trend for *FCGR3A*-158V patients to have worse outcomes, particularly CRR at 7 months (41% VV vs 59% FF/VF; $p=0.07$). There was also no correlation between polymorphism status and TTNT, PFS or OS (figure 27).

Response by treatment arm was considered to assess the impact of rituximab maintenance (table 7). Those randomised to R4+M had higher ORR and CRR than those randomised to R4, which was highly significant by month 25 (ORR (CRR) R4: 73% (44%); R4+M: 81% (56%); $p=0.03$ (0.01)). There was no significant interaction for the *FCGR3A* polymorphism. When looking at response by polymorphism results, each genotype had at least a trend towards better response rates when treated with R4+M than with R4, at every time point. As proportionately fewer VV patients were randomised to R4+M than VF/FF patients (52% vs. 72%), weighting calculations were performed to give theoretical response rates if 72% VV patients had been allocated maintenance (table 8). ORR (CRR) would be 78% (46%) and 82% (63%) at 7 and 13 months, respectively, still no better than FF/VF patients. At 25 months, VV patients would be expected to have slightly better response rates than FF/VF patients; however, these are still not significantly different (ORR (CRR) VV: 81% (72%); FF/VF: 72% (63%); $p=0.53$ (0.36)).

As there are relatively small numbers of VV patients, results may be disproportionately swayed by a few random events. Transformation occurred in 2/31 (6%) VV patients who received rituximab and 5/228 (2%) VF/FF patients. On the other hand, 4/228 (2%) of VF/FF patients but no VV patients died of second malignancies. A predictive effect of V homozygosity on response was not seen even when patients with histological transformation were excluded.

Baseline tumour bulk

The range of the products of the bidimensional measurements of the largest baseline involved lymph node was 84-4485mm², with a median value of 448mm². *FCGR3A*-158V homozygotes were significantly more likely to have LB at baseline compared with VF/FF patients (VV: 20LB, 11HB; VF/FF: 107LB, 121HB; p=0.08) (table 3). CRR was significantly better for LB patients than HB patients (67% vs. 45% at 7months: p=0.0004; 71% vs. 51% at 25 months: P=0.001) whilst the difference in ORR was less marked and did not reach statistical significance (83% vs. 80% at 7 months: p=0.63; 77% vs. 63% at 25 months: p=0.01)(tables 9A and 9B; figure 28). The PFS event rate was 26% for LB and 34% for HB patients (p=0.22) whilst the proportion starting new treatment was 16% and 23%, respectively (p=0.06)(table 10; figure 29). There was no interaction between polymorphism status and the effect of disease bulk on response or event rates.

FCGR2A-H131R and FCGR2B-I232T

The observed polymorphism frequencies for *FCGR2A*-H131R were 53(21%) HH, 142(55%) HR and 61(24%) RR and for *FCGR2B*-I232T, 212(83%) were II, 42(16%) IT and 2(1%) TT. There were no significant differences in the baseline characteristics by polymorphism in terms of age, gender, stage, performance status marrow involvement and FLIPI score (table 4). For the *FCGR2A*-H131R polymorphism, the difference in proportion of patients randomised to each arm approached significance when comparing HH with HR/RR (79% vs 67% randomised to R4+M, respectively, p=0.09).

There was no significant difference in ORR or CRR by either polymorphism at 7, 13 or 25 months (table 5). This was true comparing heterozygotes separately or combined with either group of homozygotes (tables 11 and 12). There was also no correlation between polymorphism status and TTNT, PFS, or OS (figure 30).

As previously stated, rituximab maintenance significantly improved overall CRR and ORR rates. When looking at response by polymorphism results and randomisation

arm, each genotype had better response rates when treated with R4+M than R4, at every time point (table 7). The single exception was *FCGR2A*-131R homozygotes at month 7, in which CRR was slightly lower with maintenance. It did appear that patients with the *FCGR2A*-HH genotype derived greater benefit from maintenance R due to a low response rate in the 12 HH patients who only received R4. Maintenance rituximab seemed to confer less response benefit in *FCGR2B*-T allele bearers with 25 months CRR 50% vs. 67% ($p=0.31$) and ORR 67% vs. 73% ($p=0.81$) in R4 and R4+M patients, respectively.

<i>FCGR3A-V158F</i>					<i>FCGR2A-H131R</i>					<i>FCGR2B-I232T</i>				
	FF (n=111)	VF (n=117)	VV (n=31)	P	HH (n=53)	HR (n=142)	RR (n=61)	P	II (n=212)	IT (n=42)	TT (n=2)	P		
Median age (years)	59	60	55		59	60	57		59	60	57			
Male gender N(%)	54 (49)	55 (47)	14 (45)	0.93	25 (47)	69 (49)	28 (46)	0.94	103 (49)	18 (43)	1 (50)	0.79		
Stage N(%)	II	30 (27)	19 (16)		11 (21)	33 (23)	13 (21)		44 (21)	12 (29)	0 (0)			
	III	37 (33)	54 (46)	0.22	22 (42)	53 (37)	26 (43)	0.97	88 (42)	13 (31)	1 (50)	0.61		
	IV	44 (40)	45 (38)		19 (36)	56 (39)	24 (39)		80 (38)	18 (43)	1 (50)			
PS N(%)	0	98 (88)	109 (92)		46 (87)	130 (92)	57 (93)		191 (90)	39 (93)	2 (100)			
	1	13 (12)	9 (8)	0.48	7 (13)	12 (8)	4 (7)	0.44	21 (10)	3 (7)	0 (0)	0.77		
FLIPI N(%)	0/1	44 (40)	32 (28)		17 (32)	49 (35)	24 (39)		70 (33)	19 (45)	1 (50)			
	2	47 (42)	51 (44)	0.23	22 (42)	62 (44)	13 3(8)	0.20	95 (45)	12 (29)	1 (50)	0.33		
	3/4	20 (18)	33 (28)		14 (26)	30 (21)	14 (23)		46 (22)	11 (26)	0 0)			
Marrow involved N(%)	44 (40)	45 (38)	12 (39)	0.98	19 (36)	56 (39)	24 (39)	0.89	80 (38)	18 (43)	1 (50)	0.78		
Raised LDH N(%)	7 (6)	3 (3)	2 (6)	0.36	4 (8)	6 (4)	2 (3)	0.52	11 (5)	1 (2)	0 (0)	0.70		
PD Largest node Median (range)	450 (88-4485)	462 (84-3600)	312 (85-3038)		480(84-2500)	425 (84-3600)	452 (100-3000)		450 (84-4485)	408 (84-2200)	1869 (700-3038)			
Lower bulk N(%)	53 (49)	54 (47)	20 (65)	0.18	22 (42)	73 (52)	30 (50)	0.47	104 (50)	22 (54)	0 (0)	0.35		
Randomised R4	31 (28)	33 (28)	15 (48)		11 (21)	46 (32)	22 (36)		66 (31)	12 (29)	0 (0)			
Randomised R4+M	80 (72)	84 (72)	16 (52)	0.07	42 (79)	96 (68)	39 (64)	0.18	146 (69)	30 (71)	2 (100)	0.61		

Table 4: Baseline characteristics by *FCGR3A-V158F*, *FCGR2A-H131R* and *FCGR2B-I232T* polymorphisms. PS=Performance Status; FLIPI= Follicular Lymphoma International Prognostic Index; LDH=Lactate Dehydrogenase, PD=Product Dimensions (mm²). R4 = rituximab induction (4 doses at weekly intervals); R4+M = rituximab induction plus maintenance (4 doses at weekly intervals followed by maintenance dose every 2 months for 2 years). P values are derived from the chi-square test for independence. In addition, Fisher's exact test was used to compare the following: lower bulk FF/VF vs VV p=0.08; HH vs HR/RR p=0.28; randomisation arm FF/VF vs VV p=0.04; HH vs HR/RR p=0.09.

		<i>FCGR3A-V158F</i>			<i>FCGR2A-H131R</i>			<i>FCGR2B-I232T</i>		
		FF	VF	VV	HH	HR	RR	II	IT	TT
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
7 months response N(%)	CR / CRu	67 (63)	62 (55)	12 (41)	26 (53)	77 (57)	37 (62)	119 (58)	21 (55)	0 (0)
	PR	26 (24)	33 (30)	10 (35)	14 (29)	39 (28)	16 (27)	51 (25)	15 (40)	1 (50)
	SD/PD/death	14 (13)	17 (15)	7 (24)	9 (18)	20 (15)	7 (11)	35 (17)	2 (5)	1 (50)
	Unknown	4	5	2	4	6	1	7	4	0
		χ^2 I CRR p=0.11; ORR p=0.34			χ^2 I CRR p=0.65; ORR p=0.62			χ^2 I CRR p=0.25; ORR p=0.07		
		χ^2 T CRR p=0.04; ORR p=0.19			χ^2 T CRR p=0.04; ORR p=0.19			χ^2 T CRR p=0.33; ORR p=0.26		
13 months response N(%)	CR / CRu	72 (67)	66 (59)	16 (55)	26 (54)	89 (65)	38 (62)	126 (62)	26 (67)	1 (50)
	PR	22 (21)	21 (19)	7 (24)	10 (21)	25 (18)	15 (25)	42 (20)	7 (18)	0 (0)
	SD/PD/death	13 (12)	25 (23)	6 (21)	12 (25)	22 (16)	8 (13)	37 (18)	6 (15)	1 (50)
	Unknown	4	5	2	5	6	0	7	3	0
		χ^2 I CRR p=0.32; ORR p=0.13			χ^2 I CRR p=0.38; ORR p=0.24			χ^2 I CRR p=0.78; ORR p=0.46		
		χ^2 T CRR p=0.14; ORR p=0.09			χ^2 T CRR p=0.44; ORR p=0.11			χ^2 T CRR p=0.68; ORR p=0.90		
25 months response N(%)	CR / CRu	66 (65)	68 (61)	17 (61)	27 (59)	85 (63)	39 (67)	125 (63)	25 (63)	1 (50)
	PR	8 (8)	11 (10)	3 (11)	6 (13)	13 (10)	3 (5)	18 (9)	4 (10)	0 (0)
	SD/PD/death	27 (27)	32 (29)	8 (28)	13 (28)	37 (27)	16 (28)	54 (28)	11 (27)	1 (50)
	Unknown	10	6	3	7	7	3	15	2	0
		χ^2 I CRR p=0.80; ORR p=0.94			χ^2 I CRR p=0.67; ORR p=0.99			χ^2 I CRR p=0.92; ORR p=0.78		
		χ^2 T CRR p=0.54; ORR p=0.77			χ^2 T CRR p=0.37; ORR p=0.95			χ^2 T CRR p=0.79; ORR p=0.76		

Table 5: Response assessments at 7, 13 and 25 months by *FCGR3A-V158F*, *FCGR2A-H131R* and *FCGR2B-I232T* polymorphisms.

Response assessments were physician reviewed centrally, blinded to genotype results. P values are shown derived from the chi-square test for independence (χ^2 I) and chi-square test for trend (χ^2 T). CR = Complete Response; CRu = Complete Response uncertain; PR = Partial Response; SD = Stable Disease; PD = Progressive Disease.

Response at month 7 by <i>FCGR3A</i> -V158F polymorphism					
	FF/VF N	VV N		FF N	VF/FF N
CR/CRu	129	12	CR/CRu	67	74
PR	59	10	PR	26	43
SD/PD/Died	31	7	SD/PD/Died	14	24
	CRR p=0.11 ORR p=0.17			CRR p=0.12 ORR p=0.48	
Response at month 13 by <i>FCGR3A</i> -V158F polymorphism					
	FF/VF N	VV N		FF N	VF/FF N
CR/CRu	138	16	CR/CRu	72	82
PR	43	7	PR	22	28
SD/PD/Died	38	6	SD/PD/Died	13	31
	CRR p=0.42 ORR p=0.61			CRR p=0.15 ORR p=0.06	
Response at month 25 by <i>FCGR3A</i> -V158F polymorphism					
	FF/VF N	VV N		FF N	VF/FF N
CR/CRu	134	17	CR/CRu	66	85
PR	19	3	PR	8	14
SD/PD/Died	59	8	SD/PD/Died	27	40
	CRR p=0.84 ORR p=1.00			CRR p=0.59 ORR p=0.77	

Table 6: Comparison of response assessments at 7, 13 and 25 months by *FCGR3A*-V158F polymorphism, combining heterozygotes with each group of homozygotes.

Fisher's exact test was used to test for significance and the p values are shown.

CR=Complete Response; CRu=Complete Response unconfirmed; PR=Partial Response;

SD=Stable Disease; PD=Progressive Disease; CRR=Complete Response Rate;

ORR=Overall Response Rate.

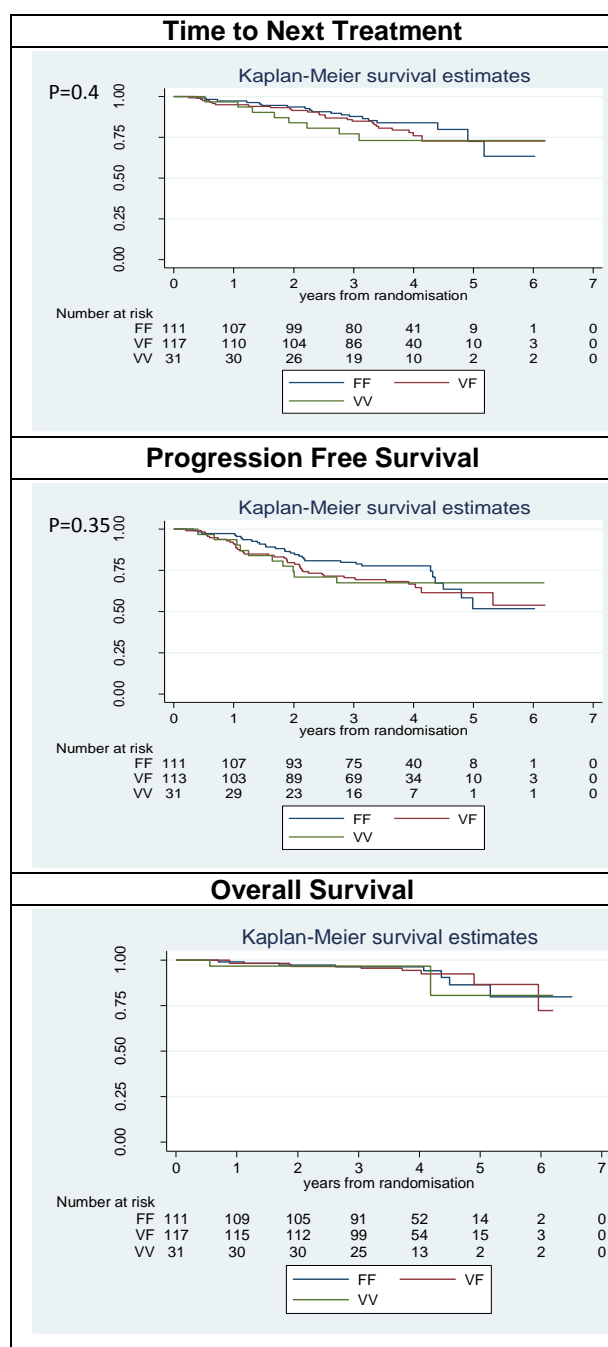


Figure 27. Kaplan-Meier estimates of time to next treatment, progression free survival and overall survival by *FCGR3A*-V158F polymorphism status. P values are derived from the log-rank test.

Results

Month 7							Month 25						
CR				OR			CR			OR			
R4		R4+M	p	R4	R4+M	p	R4		R4+M	p	R4	R4+M	p
FCGR3A-V158F							FCGR3A-V158F						
FF	18(60)	49(64)	0.73	23(77)	70(91)	0.05	13(48)	53(74)	0.03	15(56)	59(82)	0.02	
VF	15(47)	47(59)	0.25	25(78)	70(88)	0.21	14(45)	54(69)	0.02	18(58)	61(78)	0.03	
VV	4(29)	8(53)	0.18	10(71)	12(80)	0.59	5(36)	12(86)	0.01	7(50)	13(93)	0.01	
Total	37(49)	104(60)	0.08	58(76)	152(88)	0.02	32(44)	119(73)	0.0001	40(56)	133(81)	0.0001	
FCGR2A-H131R							FCGR2A-H131R						
HH	4(36)	22(58)	0.54	6(55)	34(89)	0.16	2(22)	25(69)	0.04	2(22)	31(86)	0.001	
HR	19(43)	58(63)	0.04	34(77)	82(89)	0.12	18(44)	67(74)	0.004	25(61)	73(80)	0.06	
RR	14(67)	23(59)	0.76	18(86)	35(90)	0.97	12(55)	27(75)	0.19	13(59)	29(81)	0.14	
Total	37(49)	103(61)	0.09	58(76)	151(89)	0.01	32(44)	119(72)	0.0001	40(55)	133(80)	0.0001	
FCGR2B-I232T							FCGR2B-I232T						
II	32(50)	87(62)	0.16	47(73)	123(87)	0.03	26(43)	99(73)	0.0001	32(54)	111(83)	0.0001	
II/IT	5(45)	16(55)	0.85	10(91)	27(93)	0.81	6(50)	20(67)	0.51	8(67)	22(73)	0.96	
Total	37(49)	103(61)	0.13	57(76)	150(88)	0.02	32(44)	119(71)	0.0001	40(56)	133(80)	0.0002	

Table 7. Response by treatment arm and FCGR3A-V158F, FCGR2A-H131R and FCGR2B-I232T polymorphism status. The figures in parentheses represent percentages. P values based on Chi-square tests (using Yate's correction) are given for each comparison between arms. CR = Complete Response; OR = Overall Response. R4 = rituximab induction (4 doses at weekly intervals); R4+M = rituximab induction plus maintenance (4 doses at weekly intervals followed by maintenance dose every 2 months for 2 years).

		R4	+	R4+M	*	n	=	"corrected"
								N (%)
7 month	CR	[28%(4/14)]	+	72%(8/15)]	*	29	=	13.5 (46)
	OR	[28%(10/14)]	+	72%(12/15)]	*	29	=	22.5 (78)
13 month	CR	[28%(5/14)]	+	72%(11/15)]	*	29	=	18.2 (63)
	OR	[28%(10/14)]	+	72%(13/15)]	*	29	=	23.9 (82)
25 month	CR	[28%(5/14)]	+	72%(12/14)]	*	28	=	20.0 (72)
	OR	[28%(7/14)]	+	72%(13/14)]	*	28	=	22.6 (81)

Table 8: Weighting calculations to give theoretical response rates in VV patients.

Patients randomised to Rituximab induction plus maintenance (R4+M) had better response rates than those randomised to Rituximab induction alone (R4). VV patients were significantly less likely to be randomised to R4+M than VF/FF patients (52% vs. 72%, $p=0.04$). The above calculations provide an estimate of the number of complete responses (CR) and overall responses (OR) expected had 72% of VV patients been randomised to R4+M.

Results

	CR/CRu	PR	SD	PD	dead	unknown
VV lower bulk (n=20)	10	4	3	2	0	1
VV higher bulk (n=11)	2	6	2	0	0	1
VF lower bulk (n=54)	34	9	7	4	0	0
VF higher bulk (n=62)	29	24	4	3	0	2
FF lower bulk (n=53)	41	8	3	1	0	0
FF higher bulk (n=55)	26	16	8	2	0	3
All lower bulk (n=127)	85	21	13	7	0	1
All higher bulk (n=128)	57	46	14	5	0	6

Table 9A: Response at month 7 by FCGR3A-V158F polymorphism and disease bulk.

The product of the dimensions of the largest baseline node was calculated for each patient; those below the median value were designated “lower bulk” and those above the median value designated “higher bulk”. CR=Complete Response; CRu=Complete Response unconfirmed; PR=Partial Response; SD=Stable Disease; PD=Progressive Disease.

	CR	PR	SD	PD	dead	unknown
VV lower bulk (n=20)	13	2	0	3	1	1
VV higher bulk (n=11)	5	1	1	4	0	0
VF lower bulk (n=54)	35	4	1	10	3	1
VF higher bulk (n=62)	35	7	3	13	0	4
FF lower bulk (n=53)	42	2	2	4	1	2
FF higher bulk (n=55)	25	7	4	14	1	4
All lower bulk (n=127)	90	8	3	17	5	4
All higher bulk (n=128)	65	15	8	31	1	8

Table 9B: Response at month 25 by FCGR3A-V158F polymorphism and disease bulk.

The product of the dimensions of the largest baseline node was calculated for each patient; those below the median value were designated “lower bulk” and those above the median value designated “higher bulk”. CR=Complete Response; CRu=Complete Response unconfirmed; PR=Partial Response; SD=Stable Disease; PD=Progressive Disease.

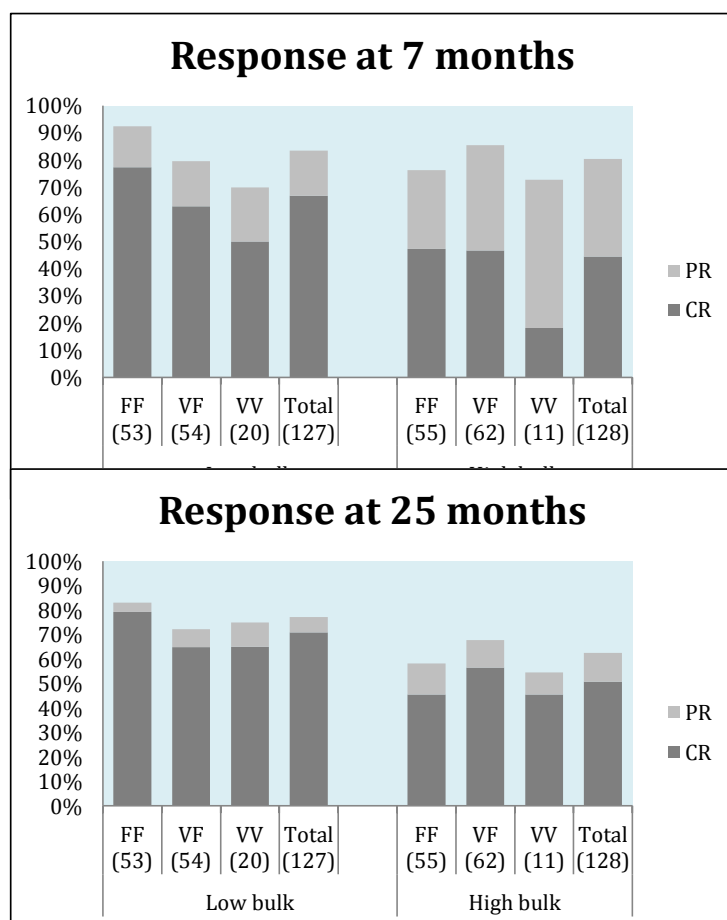


Figure 28: Response at months 7 and 25 by *FCGR3A*-V158F polymorphism and disease bulk at diagnosis. The products of the dimensions of the largest baseline lymph node for each patient were calculated; those smaller than the median measurement were designated “lower bulk” and those larger than the median designated “higher bulk”.

	Progressed/ dead	Alive, no progression		New treatment	No new treatment
VV lower bulk (n=20)	4	16		4	16
VV higher bulk (n=11)	5	6		4	7
VF lower bulk (n=54)	20	34		10	44
VF higher bulk (n=62)	19	43		14	48
FF lower bulk (n=53)	9	44		6	47
FF higher bulk (n=55)	19	36		11	44
All lower bulk (n=127)	33	94		20	107
All higher bulk (n=128)	43	85		29	99

Table 10: Number of patients suffering progression/death and commencing new treatment by *FCGR3A*-V158F polymorphism and disease bulk. The product of the dimensions of the largest baseline node was calculated for each patient; those below the median value were designated “lower bulk” and those above the median value designated “higher bulk”.

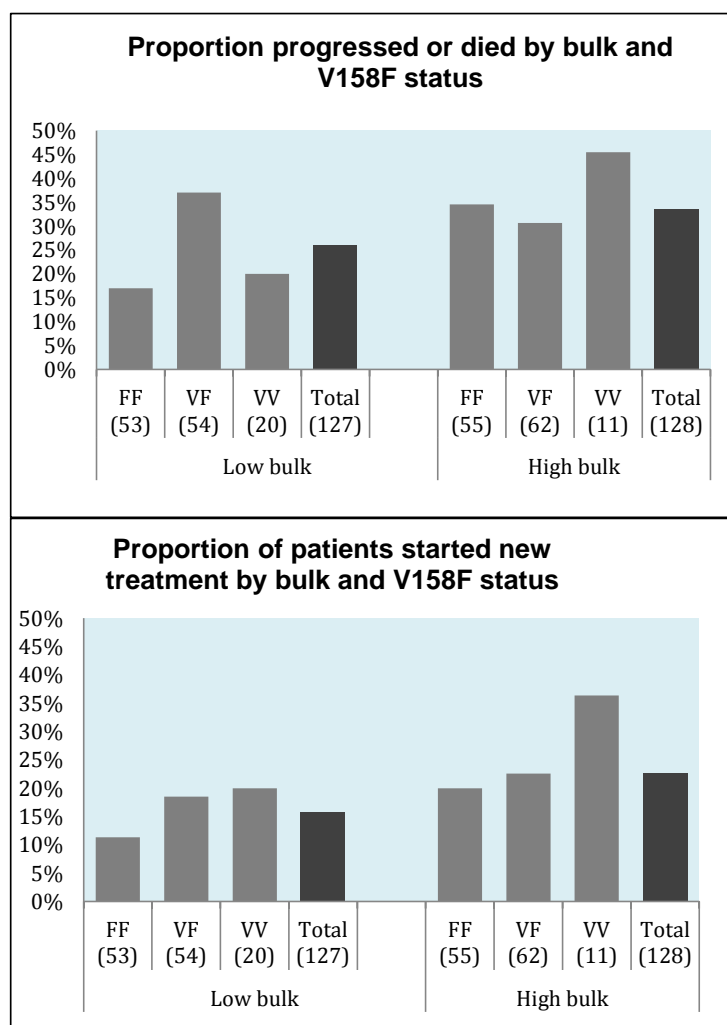


Figure 29: Proportion of patients suffering progression/death and commencing new treatment by *FCGR3A*-V158F polymorphism and disease bulk. The product of the dimensions of the largest baseline node was calculated for each patient; those below the median value were designated “lower bulk” and those above the median value designated “higher bulk”.

Response at month 7 by FCGR2A-H131R polymorphism					
	HH/HR N	RR N		HH N	HR/RR N
CR/CRu	103	37	CR/CRu	26	114
PR	53	16	PR	14	55
SD/PD/Died	29	7	SD/PD/Died	9	27
CRR p=0.46 ORR p=0.53			CRR p=0.52 ORR p=0.50		
Response at month 13 by FCGR2A-H131R polymorphism					
	HH/HR N	RR N		HH N	HR/RR N
CR/CRu	115	38	CR/CRu	26	127
PR	35	15	PR	10	40
SD/PD/Died	34	8	SD/PD/Died	12	30
CRR p=1.00 ORR p=0.43			CRR p=0.19 ORR p=0.13		
Response at month 25 by FCGR2A-H131R polymorphism					
	HH/HR N	RR N		HH N	HR/RR N
CR/CRu	112	39	CR/CRu	27	124
PR	19	3	PR	6	16
SD/PD/Died	50	16	SD/PD/Died	13	53
CRR p=0.53 ORR p=1.00			CRR p=0.50 ORR p=1.00		

Table 11: Comparison of response assessments at 7, 13 and 25 months by FCGR2A-H131R polymorphism, combining heterozygotes with each group of homozygotes.

Fisher's exact test was used to test for significance and the p values are shown.

CR=Complete Response; CRu=Complete Response unconfirmed; PR=Partial Response;

SD=Stable Disease; PD=Progressive Disease; CRR=Complete Response Rate;

ORR=Overall Response Rate.

Response at month 7 by <i>FCGR2B</i> -I121T polymorphism		
	II N	IT/TT N
CR/CRu	119	21
PR	51	16
SD/PD/Died	35	3
CRR p=0.60 ORR p=0.16		
Response at month 13 by <i>FCGR2B</i> -I121T polymorphism		
	II N	IT/TT N
CR/CRu	126	27
PR	42	7
SD/PD/Died	37	7
CRR p=0.72 ORR p=1.00		
Response at month 25 by <i>FCGR2B</i> -I121T polymorphism		
	II N	IT/TT N
CR/CRu	125	26
PR	18	4
SD/PD/Died	54	12
CRR p=0.86 ORR p=0.85		

Table 12: Comparison of response assessments at 7, 13 and 25 months by *FCGR2B*-I232T polymorphism, combining heterozygotes with TT homozygotes. Fisher's exact test was used to test for significance and the p values are shown. Due to the very small number of T homozygotes, an analysis of II/IT vs. TT was not performed. CR=Complete Response; CRu=Complete Response unconfirmed; PR=Partial Response; SD=Stable Disease; PD=Progressive Disease; CRR=Complete Response Rate; ORR=Overall Response Rate.

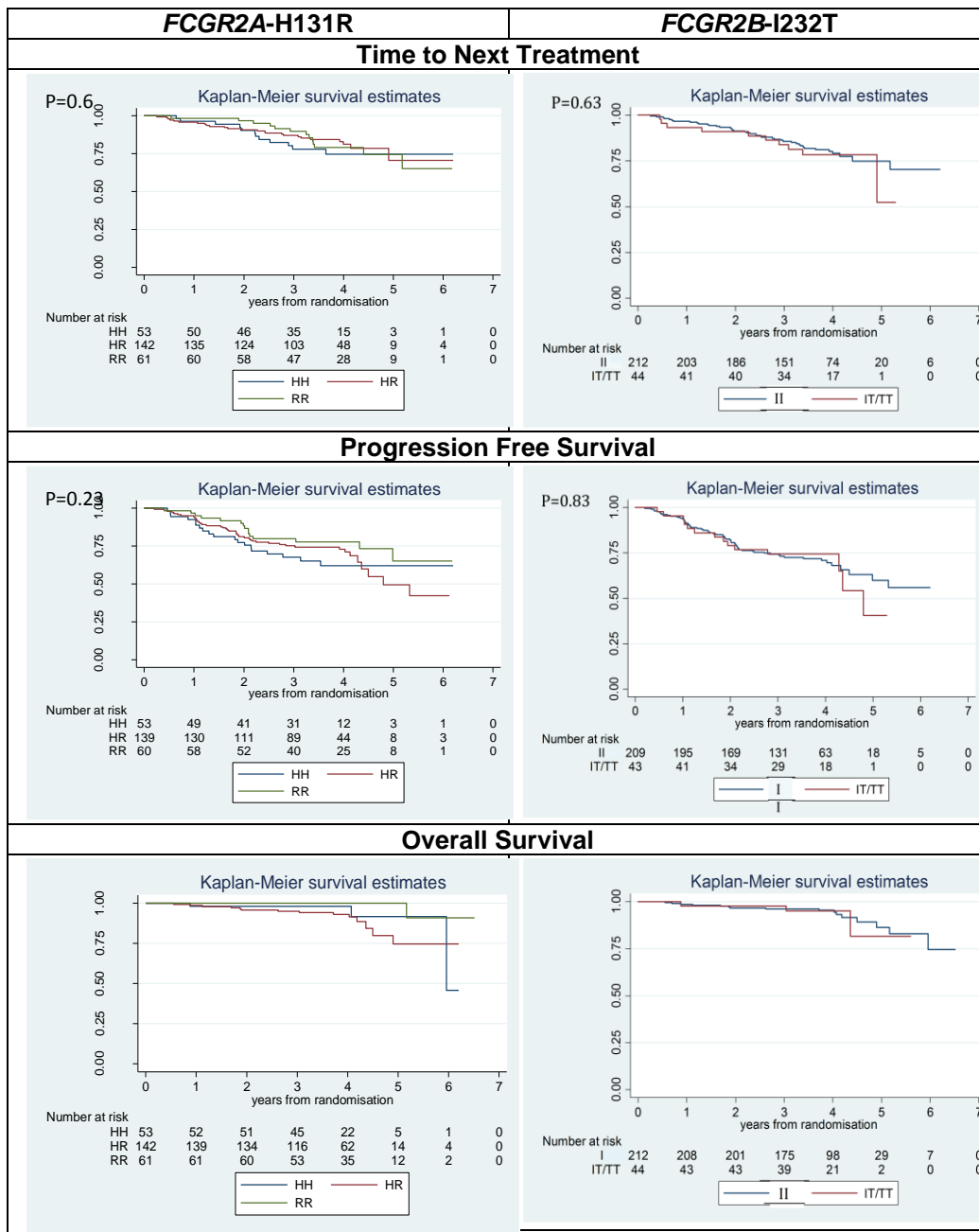


Figure 30: Kaplan-Meier estimates of time to next treatment, progression free survival and overall survival by *FCGR2A*-H131R and *FCGR2B*-I232T polymorphism status. P values are derived from the log-rank test.

C1QA-Gly70_{GGG/GG}

The observed polymorphism frequencies for C1QA-Gly70_{GGG/GGA} were AA 77(31%), AG 125 (50%) and GG 48 (19%). There were no significant differences in the baseline characteristics by polymorphism in terms of age, gender, stage, performance status marrow involvement and FLIPI score (table 13).

There was no significant difference in ORR or CRR by polymorphism at 7, 13 or 25 months (table 14). This was true comparing heterozygotes separately or combined with GG homozygotes (table 15). There is a trend at all time points for GG patients to respond more poorly than AA/AG, reaching statistical significance at month 13. There was no correlation between polymorphism status and TTNT or PFS (figure 31). As previously stated, rituximab maintenance significantly improved overall CRR and ORR rates. When looking at response by polymorphism results and randomisation arm, each genotype had better response rates when treated with R4+M than R4, at every time point (table 16).

It has previously been reported that patients with follicular lymphoma who respond to rituximab monotherapy may have a greater duration of response if they are C1QA-Gly70_{GGA} homozygotes. Therefore further analyses were carried out, looking at responders at month 7. TTNT and PFS were compared by polymorphism status in complete and overall responders to rituximab (figure 32). Again, there is no significant difference by polymorphism; however there is a trend for poorer PFS in C1QA-Gly70_{GGG} homozygotes.

Comment [LL1]: ?logrank for GG vs AG/AA for PFS in responders significant

C3-102-RG

The observed polymorphism frequencies for C3-102RG were GG 34(14%), RG 46 (19%) and RR 162 (67%). Baseline characteristics by polymorphism are shown in table 13. The groups are broadly comparable in terms of age and gender. However, RR patients have a trend to poorer performance status, more advanced stage and

more marrow involvement, and they are significantly different from GG and RG groups in terms of likelihood of higher FLIPI scores.

Comparing GG, RG and RR patients separately, difference in ORR or CRR by polymorphism did not reach significance, although there was a trend for better responses in RR patients at the earlier time points of 7 and 13 months, which was much less marked by month 25 (table 14). When comparing RR patients with GG/RG combined, the difference in CRR was statistically significant at month 7. Differences in ORR at month 7 and CRR at month 13 were of borderline statistical significance. Again, by month 25 this difference was no longer apparent (table 16). There was no significant correlation between polymorphism status and TTNT or PFS (figure 31). Any trend is for worse PFS in GG patients. As previously stated, rituximab maintenance significantly improved overall CRR and ORR rates. When looking at response by polymorphism results and randomisation arm, each genotype had better response rates when treated with R4+M than R4, at every time point (table 17). The single exception is in RG patients in which the R4+M group had slightly inferior CRR at month 7. There is no evidence of influence of C3-102-RG polymorphism status on the benefit derived from rituximab maintenance.

Results

<i>C1QA-Gly70</i> _{GGG/GGA}					<i>C3-102-RG</i>			
	AA (n=77)	AG (n=125)	GG (n=48)	P	GG (n=34)	RG (n=46)	RR (n=162)	P
Age median (range)	58 (41-82)	59(35-86)	61(29-80)		62(29-78)	58(41-82)	60(36-86)	
Male gender N(%)	36 (47)	60 (48)	24 (50)	0.94	15 (44)	23 (50)	79 (49)	0.86
Stage N(%)								
II	20 (26)	29 (23)	7 (15)		8 (24)	13 (28)	35 (22)	
III	26 (34)	51 (41)	19 (40)	0.51	16 (47)	16 (35)	59 (36)	0.58
IV	31 (40)	45 (36)	22 (46)		10 (29)	17 (37)	68 (42)	
PS N(%)								
0	72 (94)	115 (92)	41 (85)	0.27	32 (94)	45 (98)	145 (90)	0.17
1	5 (6)	10 (8)	7 (15)		2 (6)	1 (2)	17 (10)	
FLIPI N(%)								
0/1	31 (40)	45 (36)	13 (27)	0.18	12 (35)	21 (46)	54 (33)	0.03
2	31 (40)	46 (37)	26 (54)		18 (53)	20 (43)	60 (37)	
3/4	15 (20)	34 (27)	8 (19)		4 (12)	5 (11)	48 (30)	
Marrow involved N(%)	31 (40)	45 (36)	22 (46)	0.28	10 (29)	17 (37)	68 (42)	0.37
Raised LDH N(%)	4 (5)	6 (5)	2 (4)	0.95	1 (3)	2 (4)	9 (6)	0.80
PD Largest node Median (range)	418 (84-4485)	440 (88-3080)	504 (84-3600)		400 (89-2107)	532 (100-3072)	458 (84-3600)	
Lower bulk N(%)	38 (50)	62 (49)	21 (44)	0.77	20 (59)	20 (43)	80 (49)	0.40
Randomised R4	18 (23)	40 (32)	18 (37)	0.21	11 (32)	14 (30)	47 (29)	0.92
Randomised R4+M	59 (77)	85 (68)	30 (63)		23 (68)	32 (70)	115 (71)	

Table 13: Baseline characteristics by *C1QA-Gly70*_{GGG/GGA} and *C3-102-RG*

polymorphisms. P values are derived from the chi-square test for independence. In addition, Fisher's exact test was used to compare the following: Performance Status (PS) *C1QA-Gly70*_{GGG/GGA} AA/AG vs GG p=0.15; *C3-102-RG* GG/RG vs RR p=0.09; FLIPI score *C3-102-RG* GG/RG vs RR p=0.03; randomisation arm *C1QA-Gly70*_{GGG/GGA} AA vs AG/GG p=0.14. FLIPI= Follicular Lymphoma International Prognostic Index; LDH=Lactate Dehydrogenase, PD=Product Dimensions (mm²). R4 = rituximab induction (4 doses at weekly intervals); R4+M = rituximab induction plus maintenance (4 doses at weekly intervals followed by maintenance dose every 2 months for 2 years).

Results

		<i>C1QA</i> -Gly70 _{GGG/GGA}			<i>C3</i> -102-RG		
		AA	AG	GG	GG	RG	RR
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
7 months response N(%)	CR / CRu	46 (63)	71 (59)	22 (48)	15 (47)	23 (52)	101 (65)
	PR	19 (25)	32 (26)	14 (29)	12 (38)	11 (25)	38 (25)
	SD/PD/death	8 (12)	17 (15)	10 (23)	5 (15)	10 (23)	16 (10)
	Unknown	4	5	2	2	2	7
		χ^2 I CRR p=0.25; ORR p=0.26			χ^2 I CRR p=0.08; ORR p=0.10		
		χ^2 T CRR p=0.12 ;ORR p=0.12			χ^2 T CRR p=0.03; ORR p=0.14		
13 months response N(%)	CR / CRu	50 (68)	79 (65)	21 (47)	20 (63)	22 (50)	106 (68)
	PR	14 (19)	24 (20)	11 (24)	7 (22)	11 (25)	29 (19)
	SD/PD/death	9 (13)	18 (15)	13 (29)	5 (25)	11 (25)	20 (13)
	Unknown	4	4	3	2	2	7
		χ^2 I CRR p=0.06; ORR p=0.05			χ^2 I CRR p=0.08; ORR p=0.15		
		χ^2 T CRR p=0.04 ;ORR p=0.03			χ^2 T CRR p=0.17; ORR p=0.30		
25 months response N(%)	CR / CRu	49 (68)	75 (65)	24 (55)	18 (40)	26 (61)	102 (67)
	PR	7 (10)	10 (9)	5 (11)	3 (10)	7 (16)	11 (7)
	SD/PD/death	16 (22)	31 (27)	15 (34)	9 (30)	10 (23)	39 (26)
	Unknown	5	9	4	4	3	10
		χ^2 I CRR p=0.32; ORR p=0.37			χ^2 I CRR p=0.61; ORR p=0.81		
		χ^2 T CRR p=0.16 ;ORR p=0.17			χ^2 T CRR p=0.35; ORR p=0.76		

Table 14: Response assessments at 7, 13 and 25 months by *C1QA*-Gly70_{GGG/GGA} and *C3*-102-RG polymorphisms. Response assessments were physician reviewed centrally, blinded to genotype results. P values are shown derived from the chi-square test for independence (χ^2 I) and chi-square test for trend (χ^2 T). CR = Complete Response; CRu = Complete Response uncertain; PR = Partial Response; SD = Stable Disease; PD = Progressive Disease.

Response at month 7 by <i>C1QA</i> -Gly70 _{GGG/GGA} polymorphism					
	AA/AG N	GG N		AA N	AG/GG N
CR/CRu	117	22	CR/CRu	46	93
PR	51	14	PR	19	27
SD/PD/Died	25	10	SD/PD/Died	8	17
	CRR p=0.14 ORR p=0.16			CRR p=0.54 ORR p=0.83	
Response at month 13 by <i>C1QA</i> -Gly70 _{GGG/GGA} polymorphism					
	AA/AG N	GG N		AA N	AG/GG N
CR/CRu	129	21	CR/CRu	50	100
PR	38	11	PR	14	35
SD/PD/Died	27	13	SD/PD/Died	9	31
	CRR p=0.03 ORR p=0.02			CRR p=0.25 ORR p=0.26	
Response at month 25 by <i>C1QA</i> -Gly70 _{GGG/GGA} polymorphism					
	AA/AG N	GG N		AA N	AG/GG N
CR/CRu	124	24	CR/CRu	49	99
PR	17	5	PR	7	15
SD/PD/Died	47	15	SD/PD/Died	16	46
	CRR p=0.17 ORR p=0.26			CRR p=0.38 ORR p=0.34	

Table 15: Comparison of response assessments at 7, 13 and 25 months by *C1QA*-Gly70_{GGG/GGA} polymorphism, combining heterozygotes with each group of homozygotes. Fisher's exact test was used to test for significance and the p values are shown. CR=Complete Response; CRu=Complete Response unconfirmed; PR=Partial Response; SD=Stable Disease; PD=Progressive Disease; CRR=Complete Response Rate; ORR=Overall Response Rate.

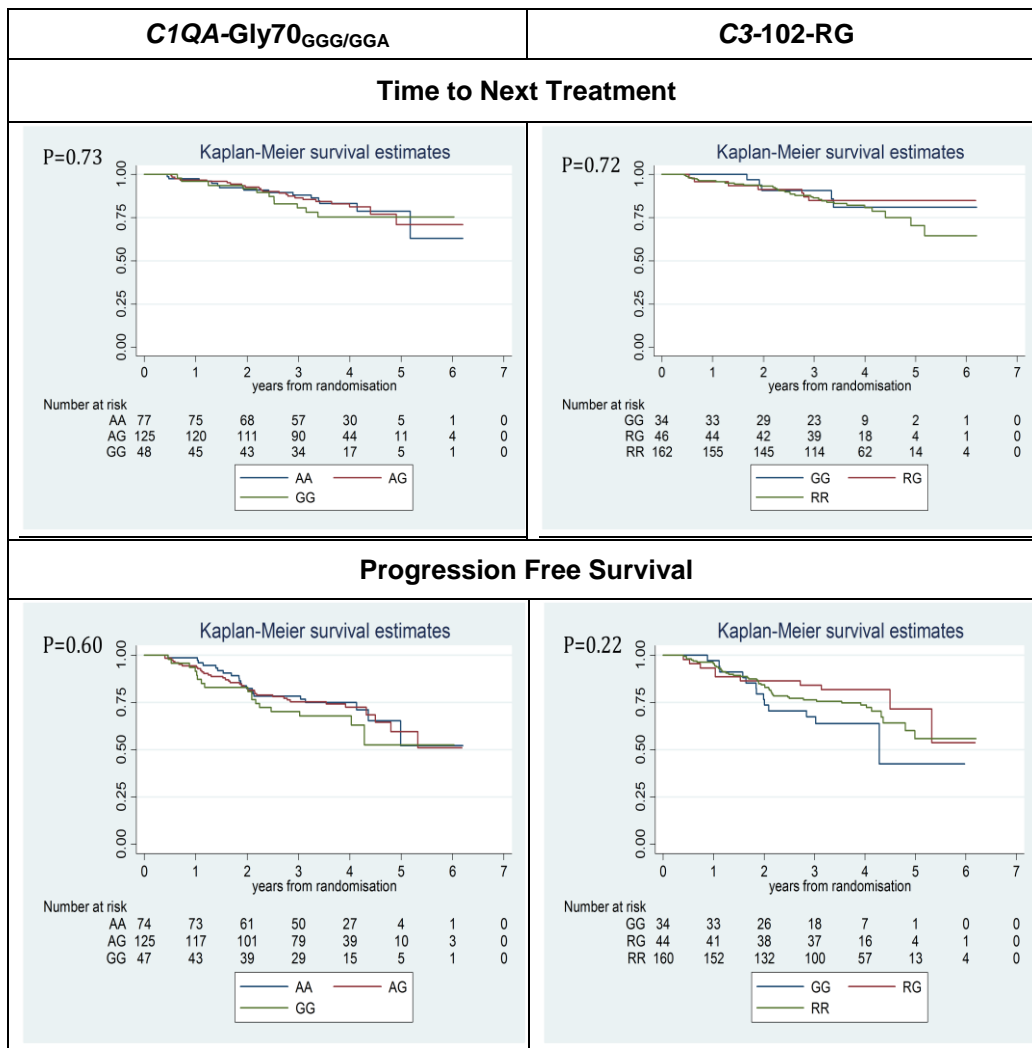


Figure 31: Kaplan-Meier estimates of time to next treatment and progression free survival by C1QA- Gly70_{GGG/GGA} and C3-102-RG polymorphism status. P values are derived from the log-rank test.

Results

Month 7							Month 25						
CR				OR			CR				OR		
R4	R4+M	p		R4	R4+M	p	R4	R4+M	p		R4	R4+M	p
CIQA-Gly70_{GGG/GGA}							CIQA-Gly70_{GGG/GGA}						
AA	10(59)	36(64)	0.90	14(82)	51(91)	0.57	6(38)	43(77)	0.008		9(60)	47(84)	0.04
AG	21(55)	50(61)	0.69	29(76)	74(90)	0.08	19(53)	56(70)	0.11		21(58)	64(80)	0.03
GG	6(33)	16(57)	0.20	13(72)	23(82)	0.67	7(39)	17(65)	0.15		10(56)	19(73)	0.38
Total	37(51)	102(61)	0.16	56(77)	148(89)	0.02	32(46)	116(72)	0.0003		40(57)	130(80)	0.0005
C3-102-RG							C3-102-RG						
GG	3(30)	12(55)	0.36	8(73)	19(86)	0.65	4(40)	14(70)	0.24		5(50)	16(80)	0.20
RG	8(57)	15(50)	0.91	9(64)	25(83)	0.31	6(50)	20(65)	0.60		8(67)	25(81)	0.57
RR	26(58)	75(68)	0.29	38(84)	101(92)	0.28	22(50)	80(74)	0.008		27(61)	86(80)	0.03
Total	37(54)	102(63)	0.24	55(80)	145(90)	0.07	32(48)	114(72)	0.002		40(61)	127(80)	0.005

Table 16: Response by treatment arm and polymorphism status. The figures in parentheses represent percentages. P values based on Chi-square tests (using Yate's correction) are given for each comparison between arms. CR = Complete response; OR = Overall Response. R4 = rituximab induction (4 doses at weekly intervals); R4+M = rituximab induction plus maintenance (4 doses at weekly intervals followed by maintenance dose every 2 months for 2 years).

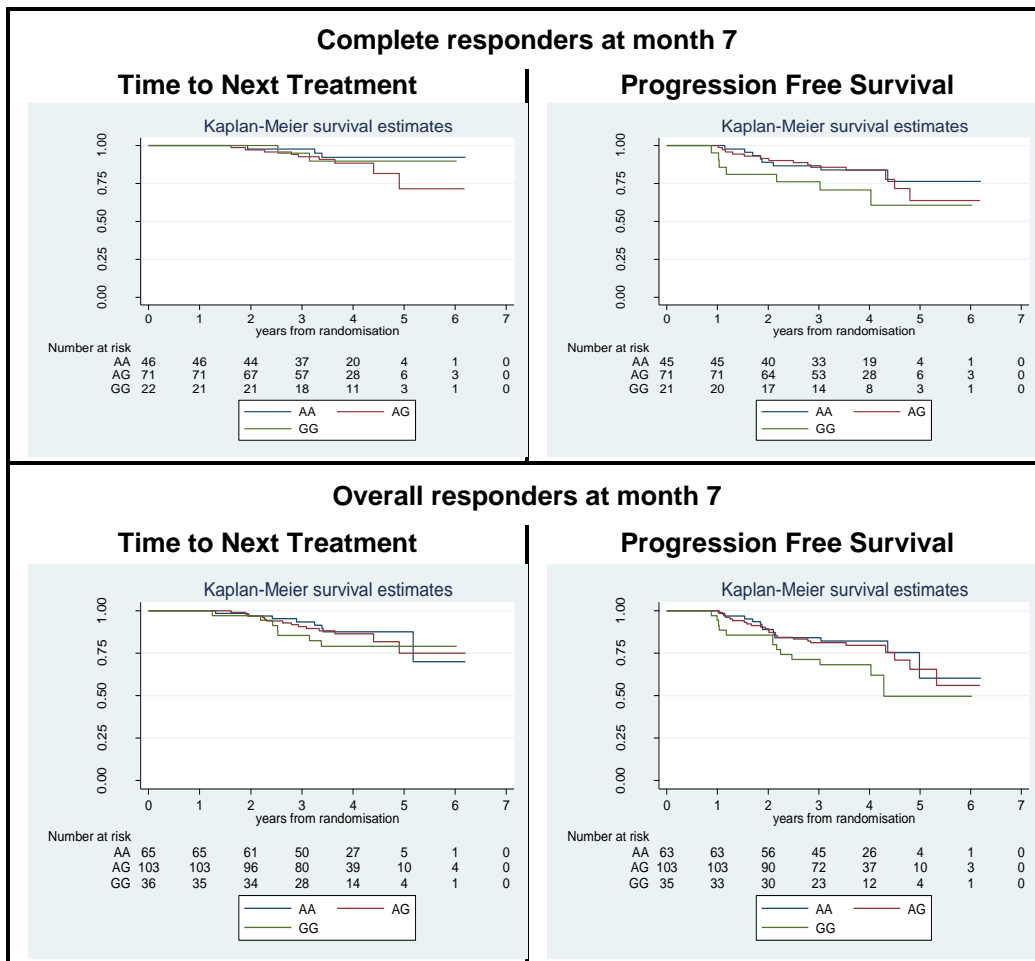


Figure 32: Duration of response in responding patients by C1QA-Gly70_{GGG/GGA} polymorphism. Comparisons of Progression Free Survival and Time to Next Treatment were made by polymorphism status for patients achieving complete and overall responses at month 7.

Response at month 7 by C3-102-RG polymorphism					
	GG N	RG/GG N	GG/RG N	RR N	
CR/CRu	15	124	CR/CRu	38	101
PR	12	49	PR	23	38
SD/PD/Died	5	26	SD/PD/Died	15	16
CRR p=0.12 ORR p=0.78			CRR p=0.03 ORR p=0.06		
Response at month 13 by C3-102-RG polymorphism					
	GG N	RG/GG N	GG/RG N	RR N	
CR/CRu	20	128	CR/CRu	42	106
PR	7	40	PR	18	29
SD/PD/Died	5	31	SD/PD/Died	16	20
CRR p=0.84 ORR p=1.00			CRR p=0.06 ORR p=0.12		
Response at month 25 by C3-102-RG polymorphism					
	GG N	RG/GG N	GG/RG N	RR N	
CR/CRu	18	128	CR/CRu	44	102
PR	3	18	PR	10	11
SD/PD/Died	9	49	SD/PD/Died	19	39
CRR p=0.54 ORR p=0.65			CRR p=0.37 ORR p=1.00		

Table 17: Comparison of response assessments at 7, 13 and 25 months by C3-102-RG polymorphism, combining heterozygotes with each group of homozygotes. Fisher's exact test was used to test for significance and the p values are shown. CR=Complete Response; CRu=Complete Response unconfirmed; PR=Partial Response; SD=Stable Disease; PD=Progressive Disease; CRR=Complete Response Rate; ORR=Overall Response Rate.

Discussion

Understanding the mechanisms of action of rituximab and the reasons for variability in clinical response could pave the way for development of more effective treatment regimens and new, more potent therapeutic antibodies. There is large body of literature derived from *in vitro* and animal studies looking at the presence, and relative contribution, of ADCC, CDC and other mechanisms in rituximab's action. However, it is difficult to know what is applicable in the clinical setting, and many of the studies throw up conflicting evidence. Differences in clinical outcomes that can be linked to random genetic variation in the immune system would provide the strongest evidence for a central role of that part of the immune system.

Our study population, derived from a multi-centre, rigorously conducted, randomised phase III trial, is ideal to explore real clinical effects of constitutional polymorphisms which have been proposed to be significant. In addition, differences in rituximab responsiveness, which could be overwhelmed by rapidly growing disease or the effects of chemotherapy, should be more easily identifiable in patients with indolent disease, treated with antibody monotherapy. However, despite the fairly large sample size, small patient numbers in some groups quickly limits interpretation of subset analysis.

***FCGR3A-V158F* polymorphism**

It has been accepted by many that the *FCGR3A-V158F* polymorphism is predictive of response to rituximab in FL and it has even been suggested that treatment decisions may be guided by genotype, with FF patients less likely to benefit from costly rituximab maintenance. However, in this, the largest study to date looking at this issue, no association has been found. Indeed, there is not even a trend in the direction of benefit for patients bearing the VV alleles.

There has been much investigation and debate concerning the relative contribution of ADCC, CDC and other mechanisms to the action of rituximab. Evidence for a

central role of ADCC comes from murine studies in which response to rituximab was abrogated in mice lacking the common Fc γ chain but preserved in mice with congenital deficiencies in complement receptors. However, there is also a substantial body of evidence for the role of CDC and other mechanisms in rituximab response. Different murine models found that mice rendered deficient in cellular effectors did not demonstrate any reduction in response to rituximab; however, complement depletion abolished it.⁸⁰ Fc γ R polymorphism studies have been taken by many to provide the most compelling evidence for a central role of ADCC in patients. There is good evidence that Fc γ R11a-158V has a higher affinity for IgG1 *in vitro* than Fc γ R11a-158F^{63,62} and patients with at least one V allele have a greater degree of NK cell activation following rituximab infusion.⁶¹ *FCGR3A*-158V homozygous patients are more prone to a number of autoimmune diseases, including SLE.⁶²

When two small studies reported improved response to rituximab in V homozygotes with lymphoma^{64,65} as predicted by laboratory research, the results generated much enthusiasm, which has translated into significant academic and commercial interest in designing new therapeutic antibodies to enhance ADCC. However, there is a risk that such enthusiasm can lead to a lack of appreciation for negative findings and acceptance of a theory without evidence from adequately powered studies. Several recent publications have assumed an impact on rituximab response by polymorphism status; when no difference is identified authors have gone so far as to interpret this as evidence that a regimen under study has the power to improve response in F-allele bearers to the level of VV.¹⁴⁵

Since the first report of improved outcomes in *FCGR3A*-158V homozygous patients, the findings have been replicated in seven other patient groups,^{65,124,90,127,134,141,213} but not in at least a further eighteen (see page 43).^{121,123,128-131,133,135-137-139,140,143-}

¹⁴⁷ Two additional studies reported a non-statistically significant trend in the direction of better outcomes for *FCGR3A*-158V allele bearers.^{132,214} However, the original study involved previously untreated patients with low-bulk follicular lymphoma treated with rituximab monotherapy. Most studies previously reporting a negative result have either included patients with more aggressive lymphoma subtypes, or

who have been treated with chemotherapy in combination with rituximab. In such patients the response directly attributable to rituximab would be expected to be less and therefore very large patient numbers would be needed to see a beneficial effect of enhanced ADCC in VV patients. Three of the eight patient groups in which the V allele has been reported to predict favourable outcome were also treated with combination immunochemotherapy for FL,²¹³ Waldenström's macroglobulinaemia (WM),¹⁴¹ and DLBCL.¹²⁷ Five remaining positive reports concern rituximab monotherapy in indolent lymphoma, and these will be considered in further detail below. Of note, three studies of *FCGR3A*-V158F polymorphism in lymphoma patients treated with chemotherapy but without rituximab have found no association with outcome.^{213,215,216}

Cartron *et al*⁶⁴ studied 49 patients with FL, previously untreated, with low bulk disease by GELF criteria. *FCGR3A*-158V homozygous patients (n=10) had significantly improved overall and complete response rates at 2 months and 12 months following rituximab induction, and a trend to improved PFS, which was not significant, when compared with *FCGR3A*-158F carriers. The patients are stated to be not different by sex, disease stage, marrow involvement or number of extranodal sites involved and *FCGR3A*-158V genotype was the only predictive factor for response by logistic regression (relative risk response at 12 months 1.7; 95% CI 1.2-2.5; p=0.03).

Weng and Levy⁶⁵ studied 87 patients with FL, the majority of which had had previous chemotherapy and half of which are described as having bulky disease. Baseline characteristics were similar by polymorphism; VV patients had only slightly less chance of bulky disease (46% vs. 54% in F carriers). Response rates between 1 and 12 months and PFS were significantly better in *FCGR3A*-158V homozygotes (n=13) and this genotype emerged as the strongest predictor of response by logistic regression analysis (OR 12.25; 95%CI 1.35-111.16). Interestingly, the next strongest predictor was *FCGR2A*-131H homozygosity. Although this finding has been replicated¹⁷⁷ there has been less acceptance of it as a true effect; again there have been many negative studies and it has been suggested that the effect may be due to genetic linkage with *FCGR3A*-V158F.^{134,128,129,178,179} The fact that the scientific rationale for the effect of the polymorphism is less convincing (H

homozygotes having an increased affinity for IgG2 rather than IgG1) may contribute to the lack of acceptance of the results, compared with the FCGR3A evidence.

Cornec *et al*¹³⁴ studied a small group of 37 FL (12 previously treated) and 13 marginal zone lymphoma (MZL) patients and found CR rates of 100% in the six VV patients (95% CI 51.7-100%) and 52.3% in F carriers (95% CI 36.9-67.3%); $p=0.02$. The baseline characteristics are stated to be comparable. In 58 patients with WM and an overall response rate of 26% to rituximab monotherapy, Treon *et al* found a response benefit for V carriers (36% vs. 9% in F homozygotes; $p=0.03$).¹²⁴ More recently they reported a similar effect in patients treated with rituximab in combination, with an ORR of 73%.¹⁴¹ Attainment of CR or very good partial response was seen in 45% of patients with at least one V allele and 9% of F homozygotes. There have been no other studies of FCGR polymorphisms in WM.

Prior to our current data, the largest study of FCGR polymorphisms in FL treated with rituximab monotherapy was the SAK 35/98 study, with 151 patients. Independent predictive factors were sought by a stepwise multivariate analysis procedure. In 2005 it was reported that *FCGR3A* genotype impacted upon EFS but not response rate (this paper included a smaller number of patients with mantle cell lymphoma).⁹⁰ Independent predictive factors identified by multivariate analysis included tumour bulk and lymphocyte count at randomisation when genotype was not included in the analysis; when genotype was included and selected by stepwise procedure, bulk and lymphocyte count were no longer selected. This may suggest some correlation between “favourable” genotype and lower bulk disease and/or lower presenting lymphocyte counts. With longer term follow-up of the same cohort genotype was no longer independently predictive of EFS.¹²⁶ Patients with at least one copy of the V allele were reported to have improved response at 2 and 12 months compared with F homozygotes; this data has been published in abstract form only.¹²⁵

If the *FCGR3A*-V158F polymorphism is predictive of response to rituximab, it is likely that response to other IgG1 monoclonal antibodies may be similarly impacted. Trastuzumab (Herceptin), a humanized anti-HER2 IgG1 monoclonal antibody, has efficacy against HER2 positive breast tumours. In vitro, increased ADCC has been

noticed in FCGR3A-158V homozygotes and FCGR2A-131-H homozygotes.²¹⁷ Two small, underpowered studies (in 54 and 50 patients respectively) reported that the predicted clinical correlation of improved outcomes did in fact occur.^{217, 218} However, a much larger study in 1218 patients did not find a clinical correlation.²¹⁹

Cetuximab is a chimeric IgG1 monoclonal antibody directed against the extracellular domain of Epidermal Growth Factor Receptor (EGFR), used in metastatic colorectal cancer and head and neck cancers. Data regarding the use of FCGR2A and FCGR3A polymorphisms to predict the response to cetuximab are inconsistent. Three retrospective studies (in 69, 52 and 106 patients respectively) report that the V/V genotype is the most beneficial FCGR3A genotype^{182, 220, 221} whereas three other retrospective studies (in 39, 65 and 58 patients each) report that the F/F genotype is the most beneficial.^{181, 222, 223} These inconsistent findings suggest that FcγR polymorphisms are not currently useful predictive biomarkers of response to cetuximab.

The absence of conclusive evidence of a significant clinical effect of the FCGR3A-V158F polymorphism on response to rituximab was the foundation for the current study. The results are negative for response at any time point, PFS, TTNT and OS. Indeed, any trend towards a difference by polymorphism is in the opposite direction than previously reported, with VV patients doing worse. This study as part of a rigorously conducted, multi-centre, randomised trial, including more patients than any positive report, suggests that polymorphisms are not implicated in actual clinical responses to rituximab monotherapy in low bulk follicular lymphoma, despite the convincing scientific rationale and *in vitro* data. We have examined potential sources of bias, which could have given an erroneous negative result.

Patients with the VV genotype did not have less favourable baseline characteristics in terms of age, gender, stage, marrow involvement, FLIPI score or performance status; indeed they had slightly more favourable characteristics (see page 89). However, randomisation to maintenance therapy or induction alone was not equal across the polymorphism groups. A smaller proportion of VV patients were allocated to R4+M (16/31 (52%)) compared to those with at least one F allele (164/228 (72%)); $p=0.04$. As maintenance therapy improved response rates, particularly at month 25,

as well as PFS and TTNT in the entire cohort, this is a potential source of bias. However, sub-analyses by treatment arm do not suggest this to be the case (page 93) and calculated theoretical response rates if the same proportion of VV patients as FF/VF had been allocated to R4+M were still not superior to FF/VF (page 94).

Serum rituximab concentrations during therapy correlate with response, and correlate inversely with baseline disease bulk.⁹⁵ In *in vitro* ADCC assays, increasing the rituximab concentration eventually overcomes the difference in cytotoxicity observed when using NK cells from *FCGR3A*-158F homozygotes or *FCGR3A*-158V homozygotes.⁵⁹ It is therefore possible that patients with very low bulk disease may achieve very high rituximab concentrations in their serum and that this would negate any potential difference in tumour kill related to *FCGR3A*-V158F polymorphism status. Although the seminal paper by Cartron *et al* included only patients with low bulk disease by GELF criteria,⁶⁴ it is possible that the patients in our cohort had lower average tumour burden. The patients in the current study would all have been managed expectantly if no trial were available. If this were the explanation for the negative findings in the current study, one would expect to see the higher bulk FF patients doing particularly badly, with less influence of bulk in VV patients; this is not the case. Although patients with higher bulk disease had lower CR rates and higher change of progression or requiring new treatment, there was no interaction with polymorphism status (see pages 95-98).

As there are relatively small numbers of VV patients, results may be disproportionately swayed by a few random events. Transformation occurred in 2/31 (6%) VV patients who received rituximab and 5/228 (2%) patients with at least one F allele. On the other hand, 4/228 (2%) of VF/FF patients but no VV patients died of second malignancies. A predictive effect of V homozygosity on response was not seen even when patients with histological transformation were excluded.

Although this is one of the biggest reported series investigating the influence of *FCGR3A*-V158F polymorphisms on rituximab response in lymphoma, a bigger study would have greater power to detect a difference, which may have been missed here. This study was not primarily powered to look at polymorphism result; rather it was powered to detect differences in clinical outcomes between rituximab monotherapy

and watchful waiting. However, this study has >90% power to detect a 15% increase in ORR at 7 months of V allele bearers over F homozygotes, at 5% significance. The statistical power falls to around 70% when comparing V homozygotes to F allele bearers, so a real difference may not have been detected. However, any trend towards better response is actually in the opposite direction; hence it is unlikely that a lack of statistical power underlies the negative results.

Since this work began, a further large series has been published, reporting no association between outcomes and polymorphism status. The PRIMA investigators reported on 460 patients from within a larger, randomised controlled trial, treated with up-front immunochemotherapy plus or minus rituximab maintenance.¹⁴³ After induction therapy, CR was observed in 65%, 67%, 66% ($p = 0.86$) of patients with *FCGR3A*-VV, VF, FF genotypes, respectively. After 2 years of maintenance therapy, response rates or PFS were found not to be influenced by *FCGR* genotype. (However, *FCGR3A* polymorphisms were found to be associated with the risk of developing grade 3 – 4 neutropenia during treatment. This is consistent with other reports;^{214, 133} Fc-FcR mediated immune mechanisms mediated may well play a role in rituximab-induced neutropenia.)

It is a distinct possibility that previous reports on this subject have been influenced by small study bias. As well as a lack of power to adequately address the issue, it has been argued that small studies are more prone to reporting bias, with positive studies reported and negative studies remaining unpublished.²²⁴ Where studies are embedded in randomised controlled trials with pre-specified protocols and trial outcomes, the risk of publication and selective reporting bias is reduced. Of those studies described above reporting a positive association, only the SAK 35/98 trial was part of a randomised study.

It has also been assumed that the genetic variation in question has no bearing upon the natural history of the disease, independent of treatment. This is only supported by the fact that no difference was seen in patients treated with chemotherapy without rituximab (this being counter to the argument that the reason studies with rituximab in combination with chemotherapy failed to show an association was because the power to detect a subtle difference related to the rituximab was reduced). No studies

have looked at the polymorphism in untreated individuals. The current data hint at a possible explanation for better outcomes in VV patients in other studies; VV patients with FL may be more likely to present with less bulky disease. Clinical follow-up of patients from the same trial, randomized to a watchful waiting approach, may provide further insight.

In conclusion, no evidence has been found that Fc γ R polymorphism status can be used to predict clinical response to rituximab therapy in FL. Considerable doubt has been cast on the assumption that increasing the strength of Fc: FcR γ interactions in future generations of therapeutic monoclonal antibodies will improve clinical response. It is perhaps unwise to interpret a lack of difference in response by polymorphism as evidence of better therapies for F allele bearers.

***FCGR2A-H131R* polymorphism**

In this cohort, *FCGR2A-H131R* polymorphisms had no effect on clinical outcome and this negative result cannot be accounted for by differences in baseline characteristics, tumour bulk, or randomisation arm. Indeed, the *FCGR2A* -131H homozygotes previously reported to have better response were somewhat more likely to be allocated to the more effective therapy, R4+M, but still did not fare better than *FCGR2A*-131R allele bearers. Statistically, the HH patients seemed to derive more benefit from rituximab maintenance than R allele bearers, but this was due to poor response in the 12 HH patients treated with R4, rather than improved response in the R4+M group. It is difficult to propose a plausible explanation for such an effect and it seems highly likely to be related to the small subset sample size.

Whilst two small studies suggested an influence of the *FCGR2A-H131R* polymorphism on response to rituximab in indolent lymphoma, there are more published negative reports than positive (see page 45). This is perhaps unsurprising as the polymorphism primarily affects strength of IgG2 binding, whereas rituximab is an IgG1 antibody. The polymorphism also affects strength of CRP binding in vitro and may have other effects not clearly elucidated which could conceivably influence

response to biological therapies in cancer. However, the current study adds a sufficient weight of evidence to conclude that there is no clinically meaningful effect on rituximab response.

FCGR2B-I232T polymorphism

The *FCGR2B*-232T allele exerts a reduced down-stream inhibitory signal and has been linked to increased incidence of several autoimmune diseases, as well as better outcome from severe malaria.⁵¹ Indeed, it has been suggested that a reduced risk of death from malaria conferred by the T allele has led to the increased frequency in certain racial groups, in part explaining ethnic differences in susceptibility to SLE.⁵¹ It has been proposed that such reduced inhibitory signal would increase rituximab-mediated tumour killing in lymphoma; however a previous study did not find any effect.¹⁸⁸ That study included 101 patients, of whom 15 were heterozygotes and only 2 T homozygotes. Our study in 256 patients identified 42 heterozygotes but again only 2 homozygotes. There is no suggestion of an effect on rituximab response from the current data. However, the number of T homozygotes included is clearly too small to draw a definite conclusion that there is no effect. It would be interesting to perform a similar study in a more racially diverse group, which would likely have a higher frequency of the minor allele.

C1QA-Gly70_{GGG/GGA} polymorphism

Results from the *C1QA*-Gly70_{GGG/GGA} polymorphism, whilst not highly statistically significant, are in line with a previous publication⁸³, and deserve further consideration. There is a trend for poorer response in G homozygotes at all time points, reaching statistical significance at month 13, and a slight trend for poorer PFS in GG patients. The trend for poorer PFS in G homozygotes is stronger when only complete or overall responders to therapy are considered. G homozygotes have, on average, higher serum c1q levels than A homozygotes. It was proposed by Racila *et al*/ that, following rituximab-mediated tumour-cell killing, apoptotic bodies are

opsonised by c1qa, with higher levels of the complement component leading to more effective removal.⁸³ In patients with lower levels of c1q, tumour debris would be expected to persist for longer, allowing more time for the development of cellular and humoral immunity. Our results looking at the interaction of this polymorphism with tumour burden are intriguing – the poor prognostic significance of higher baseline tumour burden is more marked in GG patients than AA or AG. Whilst the numbers in each group are small and must be interpreted with caution, it is possible that the favourable genotype is able to abrogate the negative impact of higher tumour burden. However, if the proposed mechanism is correct, it seems unlikely that this would solely relate to rituximab therapy; lymphoma cells, and indeed other malignancies, killed by other means would also provide more fragments for dendritic cell processing and presentation when less effectively removed from the circulation. Racila *et al* have also reported an increased rate of metastasis in breast cancer patients bearing the G allele compared with A homozygotes.¹⁹⁵

C3-R80G polymorphism

Whilst the C3-R80G polymorphism does not seem to impact on TTNT, PFS or late responses to rituximab, there is a significant difference in early responses. R homozygotes had better responses, and this was despite their slightly unfavourable baseline characteristics. It is known that complement activation occurs shortly after rituximab infusion *in vivo*. A functional difference in the two C3 alleles is strongly suggested by its association with various disease states, although the mechanism of such a difference is not clear.^{199,200,202} It may be that the complement pathway exerts disease control early in rituximab therapy and inter-individual differences in CDC affect this early response. Other mechanisms such as ADCC and vaccinal effects may be more important later in the treatment course and in maintaining remission.

Conclusions

It is clear that none of the five polymorphisms studied could be used to select a group of patients who would not respond to rituximab or derive benefit from maintenance therapy. It seems likely that the response to rituximab in a given individual is influenced by a wide variety of factors, including tumour bulk and histology, serum rituximab level achieved and genetic factors including polymorphisms in various complement components and FcγRs. Rituximab seems to exert effects by various mechanisms, and the effect of genetic differences in one effector mechanism are likely compensated for in other areas, precluding the detection of large clinical outcome differences.

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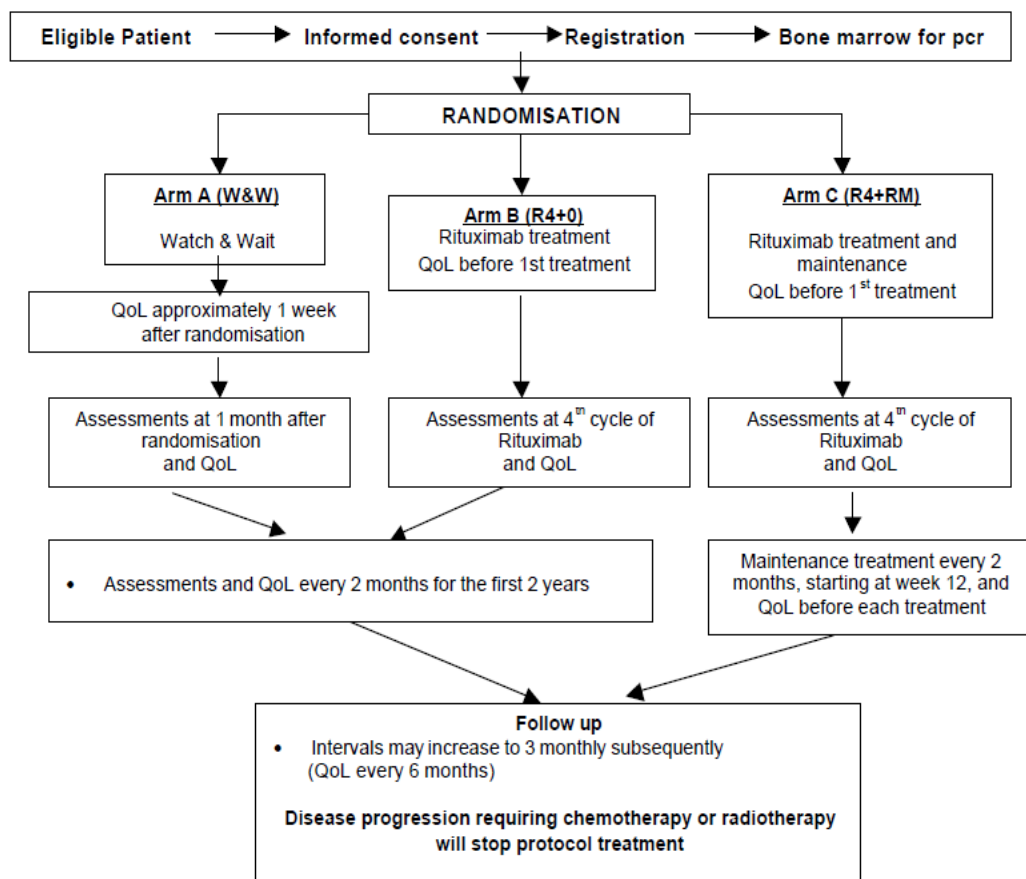
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Appendix 1 – Schema for the randomised trial



Appendix 2 – Randomisation requirements

Inclusion criteria:

- Minimum age 18 years, no upper age limit
- Follicular lymphoma grade 1, 2 and 3a
- Stage II, III and IV disease
- Asymptomatic without “B” symptoms or severe pruritus
- Blood count: Hb > 10g/dl, Neuts > 1.5×10^9 /l, Plts > 100×10^9 /l and < 5×10^9 /L circulating tumour cells
- Renal function: Creatinine < 2 x ULN for reasons other than lymphoma
- Normal liver function (bilirubin (must not exceed 30 μ mol/l), alanine transaminase/aspartate transaminase, alkaline phosphatase)
- Low tumour burden, defined as:
 - LDH within normal range
 - Largest nodal or extra nodal mass less than 7cm
 - No more than 3 nodal sites with a diameter more than 3cm
 - No significant serous effusions detectable clinically or on CT (small, clinically non-evident effusions on CT scan are not deemed significant)
 - Spleen enlargement less than or equal to 16cm by CT
- No other (non-lymphoma) immediately life-threatening disease
- Must have disease measurable in at least 2 dimensions either clinically or radiologically
- Entry within 3 months of biopsy with no prior therapy
- ECOG Performance status 0-1
- No evidence of histological transformation
- No second malignancy unless treated with curative intent and patient has been disease free for >2 years
- If second malignancy was basal cell carcinoma or squamous cell carcinoma of the skin or carcinoma of the cervix, the patient need only be currently disease free
- Patient must be able to give informed consent
- No critical organ failure or organ compression (e.g. ureteric obstruction)

Exclusion criteria:

- Patients known to be HIV positive
- Patients with Hepatitis B infection.
- Pregnant or breast feeding patients
- Women of childbearing potential not willing to take adequate contraceptive precautions if randomised to rituximab containing arm

Baseline assessment:

1. Full history and physical examination with site and dimensions of palpable lesions recorded (a maximum of 6 target lesions should be identified: see response criteria, appendix 3).
2. Measurement and documentation of spleen and liver size and ENT examination if indicated.
3. CT scan chest, abdomen and pelvis with measurement of nodal and extra nodal masses. Neck CT if cervical lymph nodes palpable. Ideally scans should be within 8 weeks of registration. If there was any clinical progression of the disease in the intervening period then an up to date scan should have been performed. MRI, PET, bone scan, ultrasound scan if indicated (not mandatory).
4. Bone marrow and tumour biopsy for histological classification according to WHO classification (bone marrow biopsy could be performed after consent and registration but before randomisation to prevent need for repeat biopsy).
5. Full blood count with differential. Blood and bone marrow for DNA and RNA extraction for bcl-2 or t(14;18) PCR analysis and Fc receptor polymorphism. Biochemistry including blood urea, serum creatinine, total protein, albumin, alkaline phosphatase, ALT or AST, LDH, total bilirubin. Serum immunoglobulin quantification and protein electrophoresis, Beta-2 microglobulin.
6. Hepatitis B serology
7. Baseline pre-randomisation questionnaire and post-randomisation (but prior to treatment) questionnaire.

Patient registration and randomisation

Following confirmation of eligibility, written informed consent and verification that baseline pre- randomisation Quality of Life questionnaire had been completed, patient details were sent to the Lymphoma Trials Office by fax. Registration was allowed prior to bone marrow examination, provided all other criteria were fulfilled. This allowed samples for PCR to be sent at the time of the initial staging marrow and thus prevented the need for a repeat bone marrow examination. Randomisation was performed once the result of the staging bone marrow was known. Patients were centrally randomised using a minimisation technique for random treatment allocation stratifying by institution and according to grade, stage and age.

Appendix 3 - Response criteria

The criteria for response evaluation according to Cheson *et al*¹³ were used.

During the initial measurement, a maximum of 6 target lesions were selected according to the following features:

- clearly measurable in at least two perpendicular dimensions
- from as disparate regions of the body as possible
- include mediastinal and retroperitoneal areas of disease whenever these sites are involved

Complete remission (CR)

1. Complete disappearance of all detectable clinical and radiographic evidence of disease and disappearance of all disease-related symptoms if present before therapy, and normalisation of those biochemical abnormalities definitely attributable to NHL for a minimum period of 2 months.
2. All lymph nodes and nodal masses must have regressed to normal size (≤ 1.5 cm in their greatest transverse diameter for nodes > 1.5 cm before therapy). Previously involved nodes that were 1.1 to 1.5 cm in their greatest transverse diameter before treatment must have decreased to 1 cm in their greatest transverse diameter after treatment, or by more than 75% in the sum of the products of the greatest diameters (SPD).
3. The spleen, if considered to be enlarged before therapy on the basis of a CT scan, must have regressed in size and must not be palpable on physical examination. However, no normal size can be specified because of the difficulties in accurately evaluating splenic and hepatic size. Any macroscopic nodules in any organ detectable on imaging techniques should no longer be present. Similarly, other organs considered to be enlarged before therapy due to involvement by lymphoma, such as liver and kidneys, must have decreased in size.
4. If the bone marrow was involved by lymphoma before treatment, the infiltrate must be cleared on repeat bone marrow biopsy of the same site. The sample on which this determination is made must be adequate (should ideally be 20 mm biopsy core).

Complete Remission uncertain (CRu [i.e. PR 75%])

Criteria 1 & 3 same as listed above for CR but with one or more of the following features:

- i. A residual lymph node mass greater than 1.5 cm in greatest transverse diameter that has regressed by more than 75% in the SPD. Individual nodes that were previously confluent must have regressed by more than 75% in their SPD compared with the size of the original mass.

- ii. Indeterminate bone marrow (increased number or size of aggregates without cytological or architectural atypia).

Partial remission

1. At least 50% decrease in SPD of the six largest dominant lesions.
2. No increase in the size of the other nodes, liver, or spleen.
3. Splenic and hepatic nodules must regress by at least 50% in the SPD.
4. With the exception of splenic and hepatic nodules, involvement of other organs is considered assessable and not measurable disease.
5. Bone marrow is irrelevant for determination of a PR because it is assessable and not measurable disease. However, if positive, the cell type should be specified in the report, e.g. large cell lymphoma or low grade lymphoma.
6. No new sites of disease.

No change or stable disease

No change or stable disease is defined as less than a PR (see above) but is not progressive disease (see below).

Progressive disease

Progressive disease (PD) (in patients who achieved PR or non responders) requires the following:

1. 50% increase from nadir in the SPD of any previously identified abnormal node for PRs or non-responders.
2. Appearance of any new lesion during or at the end of therapy.

Relapsed disease

Relapsed disease (in patients who achieved CR, CRu (PR 75%)) requires the following:

1. Appearance of any new lesion or increase by 50% in the size of previously involved sites.
2. 50% increase in greatest diameter of any previously identified node greater than 1 cm in its short axis or in the SPD of more than one node.

Appendix 4: Letter to sites requesting missing samples

CANCER RESEARCH UK & UCL CANCER TRIALS CENTRE UCL Cancer Institute

Haematology Trials Group
Cancer Research UK & UCL Cancer Trials Centre
90 Tottenham Court Road
London W1T 4TJ
Tel: +44 (0)20 7679 9283
Fax: +44 (0)20 7679 9861
Email: ll@ctc.ucl.ac.uk



LTO Director: Professor DC Linch
Centre Director: Dr J A Ledermann

<Date>

«GPname»
«Address»
«Address_1»
«Address_2»
«Post_code»

Dear Dr «GPname»

NCRI “Watch and Wait” trial in asymptomatic, advanced follicular lymphoma

Re: Patient: «Patients_name» Date of Birth: «D_O_B» Trial No: WW Trial No.

This patient was entered into the Watch and Wait trial in follicular lymphoma. A protocol requirement was the provision of blood and marrow samples at diagnosis / trial registration for bcl-2 or t(14;18) PCR and analysis of Fc gamma receptor polymorphisms, which have been linked to rituximab response. Unfortunately no samples have ever been received for your patient. However, Fc gamma receptor polymorphism status can be ascertained on any DNA-containing sample, from any time-point before or after treatment. Therefore, we asking that you provide a further sample from this patient to fully comply with the protocol. Ideally, this would be a new blood or bone marrow sample. However, if that is not possible, stored, unstained slides or tissue blocks would be acceptable as DNA can be extracted from these.

Blood (20-30ml) or bone marrow (first 2-3ml) should be added to EDTA and sent by first class post or guaranteed delivery, preferably not on a Friday or weekend. Samples should be addressed:

For the attention of Yashma Patel
UCL Cancer Institute, Department of Haematology
72 Huntley Street
LONDON
WC1E 6DD

Tissue blocks or unstained slides should also be sent to Yashma Patel at the same address.

Many thanks for your help in this matter.

Yours sincerely,

Dr Kirit Ardeshta
Chief Investigator

Professor David Linch
Clinical Trial Coordinator

Appendix 5: *FCGR3A* gene sequence - Selected section showing primer annealing and polymorphic sites.

85621	TACCAGTCCCGCCCTTCCCTCCCTGGTGGTTACATTGAGTTCTCCTTCACAATTTCTGC	85680
85681	AGCCACTCCGTGGCCACCGTCACCTTATTCCTGACTGCCACAAGAGTCTTCAATATTC	85740
85741	TTTGATTGCCTATTCCCTCTGAAATCTACCTTTTCCCTCTAATAGGGCAATTCATCATTTT	85800
85801	CAATGCAATTTTACTCTGATCTAGAACTTACTGTGAATCCTTGTACCTGCCACAGCA	85860
85861	AATCTAAGTCTAGCACTTAAGGATCCTGCAGATATGCTCATCGTTGCTTCTCACTTACCT	85920
85921	CATTGCTTAGTCCCTCTGCTCTAACCCTGTGTGTTGATCACATGTGTGTGTGCCCTCTT	85980
85981	CCCCATTAGACAAAGGCTTGGTATGACTTCAGTTCTTTCAGGGCCCCATCAGCTCTT	86040
86041	CCCCAAAGGGAGCTATGCAGGGTGTACTCCCAATCTGGCTTTCCCTTATGTCTCAGGATC	86100
86101	TGGGTGGTACGTGGCCCTTCACAAAGCTCTGCACTGAGAGCTGAGGCCTCCCGGCCTG	86160
86161	GGGTGCTGTGTCTTTTCAGGCTGGCTGTTGCTCCAGGCCCTCGGTGGGTGTTCAAGGAG	86220
86221	GAAGACCCTATTACCTGAGGTGTACAGCTGGAAGAACACTGCTCTGCATAAGGTAC	86280
86281	TATTTACAGAATGGGCAAGCAGGAAGTATTTTCATCATAATTCTGACTTCTACATTCCA	86340
	atcagattcga	
86341	AAAGCCACACTCAAAGACAGCGGCCTCTACTCTGTCAGGGGGCTTTTGGGAGTAAAAAT	86400
	gagtcgtgca	
86401	GTGTCTTCAGAGACTGTGAACATCACCATCACTCAAGGTGAGACATGTGCCACCCTGGAA	86460
86461	TGCCCAGGGACGCCTGTGTGTGGAACCTGCAATCACACTGGGAAGTTGAGTTGGGAGGAG	86520
86521	ATTCTGTATTCTTACACGCACTTCTTCATATGTGGTTCCCTCCTGGTGATCACCAGGAGG	86580
86581	TCCCCAAAAGTCCCTGATTGCAGGGTAGGTTTGCAGCTCTGTTTTCAGTCCATTCTTTTGG	86640
86641	GGTAGCTAGGAGGTGTCTTCACTCTGCAGCATGATGGCAGGAGCAGAAGCCACATCTCC	86700
86701	TCCCCAATAAATACCTCTGTCTTTCCCTTACGCTAATCACACCCACGGTGTATATGTTCC	86760
86761	TATCGTGCTGGCCTCCTTCTTATCCAAGCCTTTTAGCCACGATCCAAACTGGCAGGAGCC	86820
86821	CCTCATCCCCCTCACAGAAAGAGCCAGAACCTGGGTCTTGGCCCTGCAGCTAATTAACCA	86880
86881	TCTGACCAGAGGTGAGCCACTTAGTCTCTCTGTAACCCCAATTTCTTCTTCGTAACAAA	86940
86941	ATAAGCTGACATTTTATGGGCACCTTTTCAGTGTGCTAGACTCTGTGCTAAACAATTCTTT	87000
87001	ACATGCACCTGGTTTGTACTATCACAGTAGACCTTCACAACATGAGATAGGTAATATTTCCA	87060
87061	TTTTACAGATGAAGTAACCGAGGTGCAAAAATAAATAAAGTTTCCCTAAGGTCACAT	87120
87121	CAAAGACTTCAAAGCCTGTATATTTAACCAGTAAGTAAAGATTTGAACAAGCACTAATA	87180
87181	TCCTATGATCCCATTAAGTCTATCCACAAAACATCTCTAGGTTCTGTAGCACCAGCCTCCA	87240
87241	GAATCAGAGCTCTAGAGTGGTGTGCCCTGGACTTTCCAGTTTCACAGAATCTCTATCTGTA	87300
87301	ACTAGCCCAAGACATAAATTGTAAACAATTTGCATGTAGAAAGGCAGCAAAACACCTTTT	87360
87361	GAGATTTTGACACTACAATGCCATAATTTGTACAAAATAATTTTCATGACACTTTAACT	87420
87421	GAAAGTAAATACTCCCAAGTGGTTAGGGAAAGAGAGCAAAATAAGCAAAATGGGGTAACAT	87480
87481	GTAACAATGAGTGGATCTGGGTAAAGGATATACGAGATTAACACTATTCTGGTCATTTT	87540
87541	TTTTTAAGTTTGGAAATATATCAAATCAAGAGTTTAAAAAATGAAATGCAAAATCAAC	87600
87601	AAATTTGTCCAGTTTCTAGACCATAGCATGTGTCTGACAATTTCTTAAGTGTACACAAA	87660
87661	ACCCAGCTTACAACCTAACTTGTTAACGCTCCCTGTGACATCTCTGTCAAACAAGCAGGA	87720
87721	GCCTTTGCTCAGTGGTTTGGTGAGCTGTCTCTGCTCAGATAGCACTAAGATCAGGAACCA	87780
87781	ATGGGAGGAAGCAATACCTTTCCCCAGACTTCCCCACCATTCTTACCATTGCCTGTG	87840
87841	CTGTTGTCAAAGACTTTCTACTGGTGACCTCACTGTTTGTTCCAAATATCTGCCTTAGTG	87900
87901	ACTGTCATTTTCTTCTCTCTCACTTCTCTTAATAGGTTTGGCAGTGTCAACCATCTC	87960
87961	ATCATTTCTTTCACCTGGGTACCAAGTCTCTTCTGCTTGGTGATGGTACTCCTTTTTCG	88020
88021	AGTGGACACAGGACTATATTTCTCTGTGAAGACAAACATTCGAAGCTCAACAAGAGACTG	88080
88081	GAAGGACCATAAATTTAAATGGAGAAAGGACCCTCAAGACAAATGACCCCATCCCATGG	88140
88141	GGGTAATAAGAGCAGTAGCAGCAGCATCTCTGAACATTTCTCTGGATTGCAACCCCATC	88200
88201	ATCCTCAGGCCCTCTCTACAAGCAGCAGGAACATAGAACTCAGAGCCAGATCCCTTATCC	88260
88261	AACTCTCGACTTTTCCTTGGTCTCCAGTGGGAAGGAAAAGCCCATGATCTTCAAGCAGGG	88320

- .. SNP T→G (predicting Phe→Val)
- Outer primers
- Inner primers – lower case letters show non-annealing nucleotides
- .. Deliberate primer mismatch
- CATG⁺ Potential *Nal*III restriction site

Appendix 6: *FCGR2A* gene sequence - Selected section showing primer annealing and polymorphic sites.

4801	CTGCTTTTAGAAGTCCCA	Y	AGATATAATACCATATATTGCCTATAAGAGAATGCTCACAT	4860
4861	CTGTCATGAAGCATCTTCATTTCTGTCTGCCAGACATCATGTCAAGTTCTGTGAGTAAC	R		4920
4921	TACCTCTGAGACTGAAAAACCCTTGAATCTATCCTTACAACTTTTTCTTATCATATTT	R		4980
4981	TGTCTTTCAG	AATGGCTGGT	GCTCCAGACCCCTCACCTGGAGTTCCAGGAGGGAGAAACC	5040
5041	ATC	RTGCTGAGGTGCCACAGCTGGAAGGACAAGCCTCTGGTCAAGGTCACATTCTTCCAG		5100
5101	AATGGAAAAATCCCAGAAATTCT	C	CGTTTGGATCCCACTTCTCCATCCCAAGCAAAAC	5160
5161	CACAGTCACAGTGGTGATTACCACTGCACAGGAAACATAGGCTACACGCTGTTCTCATCC			5220
5221	AAGCCTGTGACCATCACTGT	C	CAAGGTATGGGAGTCTGCCAAGATGTAGGGAGGGGAGA	5280
5281	AGAGGGGATGGACAAGGCTGAGGTACATGGGCTTACATGGAGGTCTGAGAAAGCCAT			5340
5341	AGCAGCAAAATTGGGCACTGGAGCAAAGAGGAGTGGGGTGGAAAGCCTGGCTAAGTATTGA			5400
5401	CCAACAAGTAGGGGCCAGAGCTTGGAGCCCTCACGTCC	AG	CTAATAGGTAGTCAGGCTG	5460
5461	TTGTTTCACTTTGAAATGTAGGCCCCAGACTAAAGATGGCAGTGAAGCAGAGCTCCCTCA			5520
5521	TTGATGCAGAGGTTCCCTAAGCTCCTGGGCATTCTTAAGAGCTGAGGTTTGCTCGTTTC			5580
5581	TTCTCATGGCTCATGTTATAR	C	CATTCACTCCAGAAAGCCTGGCACGTCATGGACTGTTC	5640
5641	AAGGCTGTGCTCCATAGAGTAATGATGCCTCCAGCTATGCGAGGCTTTGGGCCACCCCTC			5700
5701	CGTACTGTCCCCAGGGG	M	TAAGGGGAATCCTTCCCTCTGCTC	5760
5761	GCTTTCATTCACTTTGGTGGAGAAACCTGGGTAGGGAGGAGGCACAAGTCCAGCCACAGAA			5820
5821	ACCCGTGTGCCAGTGAGGCTGGGGATGTGGTGAATCTTGCATTGGTGAGTGACTCAGACAC			5880
5881	AGAAGAGCTTCAGGTGACAAGCACTGGGACATAGCATTGGAGGTGGGAGGTGGGACAGGG			5940
5941	AGAATACAAACGTTGTCAATTAATAATAGTA	AC	CCCCATCCTGCCCTAATGTCTGTCTTCC	6000
6001	CTAGT	GGCCAGCATGGGCAGCTCTT	CACCRATGGGGRTCATTGTGGCTGTGGTCAATTGCG	6060
6061	ACTGCTGTAGCAGCCATTGTTGCTGCTGTAGTGGCCTTGATCTACTGCAGGAAAAAGCGG			6120
6121	ATTTTCAG	GTTTGTAGCTCCTCCCAGTCCCTTTTGTATCAGTTTCCATTTGGCCCAGGGC		6180
6181	CTAACCCAGACATTGCCAGAATCCCGCTCTTAGGGCTAGATATGCATTCCGATCTAGGC			6240
6241	CCACCTTTTATTTATTAGTTTATTATTAGTTTATCCTCAACAAGAGCACTAATAGGAGT			6300

- .. SNP G→A (predicting Arg→His)
- Primers forward MP7931 and reverse MP7932
- Substitutions in primers to create restriction sites
- cg⁺cg Potential *Bst*UI restriction sites

Appendix 7: FCGR2B gene sequence - Selected section showing primer annealing and polymorphic sites.

91621	CAAATTTTCCTCAAAAGTACACAGCAAACGAATGACCCACTGGTGACACTGCTGCCTTTA	91680
91681	GACCTGCTGGAAGAAGCTCCACATTTATTAACATTCCCAGTAATTTATCAGGTAG	91740
91741	CATTCATCAGGTAACATTTGTTGCACATTGACTTTTCTACTGTCCACAAAGGCATAT	91800
91801	GTCCTTATCATATGCGGACTCCTCGGTACACTGGATTCTTCCCTCCCTCGACATGG	91860
91861	AAGAGATGGCATCTTAGGGTCTCTGTGTTCTTCCCTGCAGAGGCTGTCGGGCAGGAAAA	91920
91921	GGCTGCAGCTGCCTTCTCGGGAGAAGGAGGAGATGAGTGTATCCTGAACACCTATTATGT	91980
91981	GCTAGGGGCTATTGTAGATACATGACACTATCATGCTATTTTCACGAATGAGGAACTG	92040
92041	AGGCTCAGAAGACTTAAATTATTTGCCCAAGAGTTCATAAATGACAGAGCCAGCATTAGA	92100
92101	GTCCAGGACTGTCTGATTTAGACCTAAGCTGTTCCCTCTGCACATCTGTGCCACCAGT	92160
92161	AAGGAAGATCTGGGCTCAGAGCTGAGCCAAGACCTCCGGGTCCTCTGCGGTTTTTTGT	92220
92221	GTCTTTTCAAGTGGCTGGTGTCCAGACCCCTCACCTGGAGTTCAGGAGGGAGAAACCA	92280
92281	TCGTGCTGAGGTGCCACAGCTGGAGGACAGCCTCTGGTCAAAGTCCACATCTTCCAGA	92340
92341	ATGAAAAATCCAAGAAATTTTCCCGTTCCGATCCCACTTCTCCATCCCAACAAGCAAAC	92400
92401	ACAGTCACAGTGGTGATTACCACTGCACAGGAAACATAGGCTACACGCTGTACTCATCCA	92460
92461	AGCCTGTGACCATCACGTGTCCAAGGTATGCCGAGTCTGCCAAGATGTAAGGAGGGGAGAA	92520
92521	GAGGGGATGGACAAGGGCTGAGGTCACATGGGCCTACATGGAGGTCTGAGAAAGGCCACA	92580
92581	GCGCAAAATTTGGGCACTGGAGCAAGAGGAGTGGTGTGGAGGCTGGCTAAGTATTGACC	92640
92641	AATGAGCAGGAGTAGGGGCCAGAGCTTGGAGCCCTCAGGTGATAGGTGACCAGGCTGTTG	92700
92701	TTCCACTTTGAAATGCAGGCCCCAGACTAAGGACGGCAGCGAAGCAGAGCTCCCTCGTTG	92760
92761	GTGCAGAGGTTCCCTAAGCTCCTGGGCATTCCCTAAGAACTGAGGTTTGCCTTTATCTTC	92820
92821	TCATGGCTCATGTTACAGCCATTCACTCCAGAAAGCCTGGCACGTGATGGACCGTTCAAG	92880
92881	GCTGTGCTCCATAGAGTAATGATGCCTCCAGCTATGCGAGGCTTTGGGCCACCCCTCCC	92940
92941	ACTGCCCTGAGGGCTAAGGGGAGCCCTTCCCTCTGTTCTGCTGCTCACAATGTACC	93000
93001	TTTATTAGTTTGGTGGAACACCTCGGTAAGCAGGAGGCATAAGTCCAGCCACAGAAACC	93060
93061	CTGTGCAGATGAGGCTGGGATGTAGTGCTGCTGCAGAAGTGAGTGACTCAGACACAGA	93120
93121	AGAGCTTCGGGTGACAAGCACTAGGACATAGCATTGGATGGGGGGAGGTGGGACAAGGA	93180
93181	GAGTACTGCCTGTCTGATGTCTGTCTTCCCTAGCTCCAGCTCTTACCAGATGGGGATC	93240
93241	ATTGTGGCTGTGGTCACTGGCACTGTCTGTAGCGGCCATTGTTGCTGCTGTAGTGGCCTTG	93300
93301	ATCTACTGAGGAAAGCGGATTCAGGTTGTAGCTCCTCCCGGTCCCTTTGGTTATC	93360
93361	AGTTTCCACTTGGGCCAGGCCCTAACCCAGACATTGCCAGAATCCCTCTCTTTGGGCTA	93420
93421	GATACACATTCAGATCTAGGCCGTATTGTATTATAGTCATTTCGTTTATTAGATCA	93480
93481	TTCAATTTGACAGACTTGAACAACTAGCTTATGTGTGAGCCAATGCGTGAGACATGGTG	93540
93541	GGTGATGCCAAGAGAAGTCCAGTCCCTGCCCTTGGGGAGTTCTCAGCATTGTGAGGAAGA	93600
93601	CAGGTCATTATAATAAGATGGGATAAGAGCAATGATAGGGACATGAAAAGCCTAGGATA	93660
93661	GAAGTACAGAGGAGGCTCCATAGCCCAACAGGGCTGAGGCAGTTGGAGAAGGTCTTGGG	93720
93721	GAGAGCAGTGTAGTGTAGTGATGGAGTGCACATAGTGGGGCCAGACTGCCTGAGTTCAAA	93780
93781	TCTAGTTCTACAGCATACCAACTATATGACCAAGTGAGTCACTCCACCTCTCTGTGCCT	93840
93841	CAGCTGTCTCAAGTGGAAAATGGGGACACTAATAGGACTTACCTCAGAGGGTTGCCATGG	93900
93901	GGATGAAAGAGGCTGATGCTCTTACACAGTGCCTGGTAGGCAGTAACAGCACACAACAG	93960
93961	GGCTGTTGCCATTCTGGAGGAGGTGGTGTGTGAGGGCAGCGCTTAGCTAGGGAAGGAGGA	94020
94021	CCCTCCAGGCACTGGGCCAAGGCAGGGATTGAAGCCGGGTGGGGCTCATATGGGTGTGCA	94080
94081	AGGGGGAGTAGAGAGGGTACTGCAAGCCACCAAGAAAGAAAGAAATCTGCTAACACCTCA	94140
94141	CAAAGCACTTTTCAATTGGTTTCATAGCCAACACCTCAGTTACTGATGATAAGTAAATACA	94200
94201	GAGAAACAGAGAGAGAGAGACTGAGACACAGGGGTGTTTTTAAAGGCAGAGCAAGCCTC	94260
94261	AGACAGATGCCATGGCGTGGACTTTCTGACACTCCTGGGCATCCCCATGGGTGAGCTGAA	94320
94321	TTCTGCCTCTGGACCAGCCCTTTTCCAGGCGGGAGCAGCCTCTGAGCAGGGGAGCTGGGG	94380
94381	GTGGGAGGACAGGAAACATCTGCCAGAGTGAAGGCCTGAGCTGGTCCCATCCAACCTTGG	94440
94441	CCCTGGTCCCTTGGTCTGAGGACTCAGGCCACCGCCTAATCCTACTAACCTCCTGTGT	94500
94501	GCCCTCCCAGCTCTCCAGGATACCCTGAGTGCAGGGAATGGGAGAGACCCTCCCTGA	94560
94561	GAAACCAGGTGAGTACAGGTTGTCTCAGGGATTGAGTATGGCTCACCAGGGCTGCCGGC	94620
94621	TGGACTGGAGCCAGGGAGAAGGGCTTGTGCAAGTTCAGCTGGGAGCCAGGGAGGGAGCA	94680
94681	GCAGTGGGAGGATGGCTGGGGCTCAAATCGCTTGGTCAGCTCTTAGTCTAACTCCTGGGC	94740
94741	CTAAAGACGACCTCTCTGGAGATGAGAAGAGAAACACCACTCCAGATACAGAAGAGAGG	94800
94801	GCTGTGTCCGAATTTCTGGTACCAGGAATCTGGTGTATTTCCAGAGCAAGAAATCAGAG	94860
94861	ATACTTTGGTCTTCTGTGGAGCTTTGGCAGAGCTGGCAAAGGATGGGGTTAGGGGCTGT	94920
94921	AAGACTGAGGCCAATAGGACGTGGGAGGCAGGAGTCTAAGGGGAAAGGAGGAGGAGCCCT	94980
94981	GGAAACCCCTTGATTGCTGAGGAATCTGCCTCTGTGGAGGGATGGGGTGGAGTGCCAG	95040
95041	GCTCAGCTGTCTGTGGAACACTAGGAGGAGGTTTGGGCTTGAGAAAATTTCTGGGGACTA	95100

95101	GGGGCACTAGTGGGCTCTACTCTGTGGGCAAACTCAGTTCTGATGCCCATGCACTCAAG	95160
95161	GTTGACTGAATTTTGGCCTGGTTCTGCCCTCATCACACAGTGGAGCCCTGAGCAAGTCAG	95220
95221	TCTCCCTGATGGGGTTCACTCTCTCACTGAGGAAATGAGTGGGTGGCCAGGGTGACCTC	95280
95281	TCAGGTCTTTCTAGATTTGACATTCTGAGAACCAGGGGCCACC	95340
95341	GGGTGTGTAAAGGAACTTCCAGGAGGATGCCAGGCAACTTCCAGACTGTAGTTAAAGTC	95400
95401	AGAGCACAAAGGAAACAGTGCCTTCTCTGGGGCTTCCATGACAGTAAGAACAGGCAGA	95460
95461	AAAAAGAGGCTGTCAAAGACCCACCTCAGATGGCTGGAGTATGATTTCAGAGCAGAGCCA	95520
95521	ACAGATTTAAGAGCTTGAGCAAAATATCACTTGTTTCAGCTGTGCTTGACGAAGGTGCGA	95580
95581	TGTCTGTCTGGCCAGGCCAGTGGTGATAGGGGCCTGTGTTACACACAGGGCCAAAACAGA	95640
95641	TCTGGAAAAACAAAGTCCAAGCAACTGGATTATCCATCTTGTGTGTGGTTCTGTCTGC	95700
95701	ACTGGCATAAGCACATTTCAAGGCACCTTTTGCAGCCAGGCATGGGGCTGCATAAATG	95760
95761	GTTTTTTTAAATCCTTTCCCAAAGGCAAGCTGGGTTGCTGGGATTCTTGGGAAATGT	95820
95821	AATAGAATGGGAATTCAGAATGCGGTTTACTGCAGAATTTACAGCATGGCAATAAAGGCT	95880
95881	CTCTATGTTTCAGGGGGTGAGAGAAGAGACTAGATGCATTGTCTCAGATGTCTCCTGACTT	95940
95941	CCTGAGAAATGCCACTTTGCAGATATGGGGCTGCTTTTCACACTGGCTAAGTCAGTTCAT	96000
96001	GGCAAAATATGAGGGAATTCAGTGTGTTTTCAGACACTAACCAAAGTCCATCCTTCCTTTC	96060
96061	TCCTTCCGTCCTCCTTCCCTCCCTTTCTCACTCACTCTCTCTTTCTTTTAAATGCCATG	96120
96121	GAGTACCATAATTTTCAAAGTTCCAACAAAGATGCACTACATTTAGACAAATGTGGAAATG	96180
96181	GCCCTCAGCCTTCTCTCCCTGAAGTCTGTGGCCAGCAAAATCTGGACTTTACTTCACTC	96240
96241	TGATGCTGAACACCCCCCTCCCCACCTCTTCCCCAATATCTCAGATCCCAAGCCTCCCT	96300
96301	GGTTTGCTTCTAGGAAAGCTCAGCAATTCCCTGAAAAGAATGCAGCTCAAGTGACTGCTC	96360
96361	CACCTTTTTCACCTTGGCCTTTGCGGAAGGCTGTGGCTGGGCTACCCATATGCATCGATCAA	96420
96421	TGAGGTCATATTTACCCAGTGCTTGGCCTAGAGGCCAAGACAGGGTCAGCAGTGCTTGC	96480
96481	TTTGGCTCTAGTTTGGGCGTTGGTTTTCAGCCTCAGCATCAGCACAGGCAGGCCCTCTT	96540
96541	CTCCAGCAGTGCTCTGGCTGATATTTCTTCTTTTCCACAGCCAATCCCACTAATCCTG	96600
96601	ATGAGGCTGACAAAGTTGGG	96660
96661	TGCCTTTTCTGTTGCCTTCTTTCTAGGCCCTCTCTGTGGATCCTTACTGCTGGTTTCTG	96720

- SNP T→C (predicting Ile→Thr)
- Previously published outer primers forward MP8233 and reverse MP8234
- Forward primer MP8235 used for patient samples
- Reverse primer MP8236 used for patient samples
- Reverse primer MP8237
- Inner primers forward MP8231 and reverse MP8232 used for patient samples
- GGGAC Potential BsmFI restriction site cuts 10 bp downstream with 4bp overhang

Appendix 8: C1QA gene sequence showing primer annealing and polymorphic sites.

1	AAATTTT	60
61	TCAAGTGATCCGCTGCCTTGACCTCCCAAAATGCTGGGATTACAGGCGTGAGCCAAAGC	120
121	ACCTAGACTTGCTGTTTTTTTAAATGTTGGCAAAAGCTAGTGACTAAGTGTTCACCATGT	180
181	GCCAAGTGTTTTGTATGTGCAAGTCATTAATAATGTTATAATAATCAGATGATATAGGACC	240
241	CCGTTCCAGTGACAGATAAACTGAGGGCTCAGGAGGTCAAGTAATTTCCCAAAGACA	300
301	TGCAACTAGGAAGCAGCAAGTCTAGAAACAGATTGCTGTGCTTCTTCTACCAATGCAC	360
361	TGTTCTTTCTTCTTTGTATTTTCCAAGCTTTTGGCAATGAATTGCATGTCACTTTTATAA	420
421	TCATGAAAAGTCCACAGGGACAGATTTTTTTAAAGATTCCCTCCGCTCTGGTGTCTG	480
481	TGCTTCGCTGGTCTCAGAACCTCACCTGCACGGCCTCCACCCACATCCTCACAGGCA	540
541	CTGGCCTCACTGCCCCACTCCCACACCAGCTGTTGCTGGGGCAGGACGCCAATGTCCC	600
601	AGTCTTGCTGAAGTCTGCTTGAATGTCCCTGGTGAGCTTCTGGCCACTGGGGAAGTTCA	660
661	GGGGGCAGGTCTGAAGAAGGGGAAGTAGGAAGGGATGTGAAACTTGGCCACAGCCTGGAG	720
721	CCACTCCCTGCTGGGCAGCCACAGGGTCCCTGGGCGAGGGCAGGAGCATCCAGTTGGAG	780
781	TTGACAACAGGAGGCAG GTGAGGCCAGAGTCCAGAGGGAGGGGGCTGCAGAGTTCTGGG	840
841	ACCCAGGGGAGCTGGC CCAGGAGGCTGGGCAGCTGAGGCAGGGAGGGAGGGAAATACTG	900
901	TGCCCTGATTCTAGGACAGGGATGGGGGTGCAGCACTGATCCAGGACCCAGAATGG GTGAG	960
961	TGGAGGGACCTTAACCTCTTGAGCCAGGGCTTGGGGGAGGGTGAGAGGCGATGAGTTAGA	1020
1021	CCTAACAACTGAAGGGTAAGACCAGGGTTCTGGGTCTTGGCCCCATAACACATAAGAAA	1080
1081	CCAGGAAAAGCCTGGGGTGCTAGAGCTGGCAGTGGGACCCAGCAGAGGGGACTGTAGGGG	1140
1141	CAGCCAGTCACCCATGCTCAGGTGGATGCTGAAAGGCGCTGAGCATGCAGAAGAGTGAGC	1200
1201	AGGTGTCGCTTTGGGCGCTTTGTGAATTCCTGTGTGTATGCAGAGCTCCACCATCTGGGTG	1260
1261	AGCCAGTGTTAATTAATAAGGAGCAGGCGCTCAATACATATTTGTTCAATGAAATCGAT	1320
1321	TACCTGTGGGAGTGATGAATGTGTGTGTCGTCAGTGAGGACAGGGTCTATTGGG	1380
1381	TATCAGTTGTGTGTCTAGGGGGGTATAGTGGATTCTGAGTTTGCTTACGTGTTTTGTA	1440
1441	GGTGCCTGGATGAGAGCTGTGTTGTGTGAGTGTGTGAAGATGTGGGTGTGCTCTGTTC	1500
1501	ATGTGTGGATGTGTGTGAGTTTGTGGTTCTGTGTATATGCGTGGGGTCTTGGGGCTGGAT	1560
1561	TGAGAGTGGACATTGAGAGCCCCAGAGGGTGATGTGCACTTGGGGAGGACTGTGCATAT	1620
1621	ATCATTTGTGTGCATGGGACTCAAGGGTGGGAGTGGGTGTGAGTGTGATGTCCA ACCTGC	1680
1681	CCAGGCCCTCCCGTGTCTCCACAGAGGCATCATGGAGGGTCCCCGGGGATGGCTGGTGCT	1740
1741	CTGTGTGCTGGCCATATCGCTGGCCTCTATGGTGACCGAGGACTTGTGCCGAGCACCAGA	1800
1801	CGGGA AGAAAGGGGGAGGCAGGAAG CCTGGCAGACGGGGCGGCCAGGCCCAAGGGGGA	1860
1861	GCAAGGGGAGCCGG TAAGCACCTTCTCTGGGACCCAGCCCTTGGACCTTGGCCTGAC	1920
1921	TTGGCCTCCAGGGTGAAGGCTTGGGGTGGCACTGAGAATCAGGAGTCCGCTTCCCCCAG	1980
1981	TGCCCCATGAATCCTCTCCAGTTTGTACTTGGCCACAGGGGCTAAGGAGGCCTAGCCTT	2040
2041	CTCGGGCCATGCTCTCAGGCCTCTCCACTCCCTGTGTACTGCCT GGGCCC TCTTTCTTCG	2100
2101	GCTTCACCTTCCCCCTCTGGAGAATGACTTGCTTTGACCAGTGAAAGTGAAAGTTCTGGC	2160
2161	AGCAATGCTGATGTTTCTCATGTTTCTTCTTGGCAGAAAAACGAGGACTCAGAGTTAG	2220
2221	ACTCAGGAGCCACTGCCTTGTGTGTGACCTCAAGCAAGTTATTGGCCCTCTCTGAGCCT	2280
2281	AAGTGGGCTCATCTGTAAAATGGAAAAATAAAGTGCCTGGCAGATAGAGAGGGATCAA	2340
2341	TAACTGAATCTGTCCCTTCACTTCTTCCAGCACCCCAACACTGTCCCTGCTTCCCCCT	2400
2401	CCCCAGCCCCCTCAGGCTTCAGAGACTCACATTCTAGAAGAAAGGCCAGGGGTAAATGGAA	2460
2461	AAGGGAAGAGGTCCTGACCAAAAGTAGAAAGGGAGTCTCATGGAATCACCTTGGGCCTCCT	2520
2521	AGTCCAAAGCAGACCAGAAGGATCACATAGACATTTGCAGTAATTTGCATCCGGGCATCC	2580
2581	TACATGTGGTACCTGAACCTGAAGCCAAGTCAAGGTCAGTCCCCAGAGGGGTATGAAGT	2640
2641	CCTCAGCCTTGGGCAGGAACACAGCAGAGCAGCCAGAGTTCCATGCATTTCCAGAACCT	2700
2701	GACACTTTCCCAAGTGCCCTGTTACACTTCTCTCCACTTTACAGATGAAGTGGAGGCTCA	2760
2761	GAGAGGTTGAGACACTTGCCCAAAGTCACACAGCCAATCAGAATCGATGCTCTGAAGGCC	2820
2821	CAGGTGCTTCATTGCCCTTTATCCCATAGACTCAGGGGGTCCAGCTCTCTCCCTGAGGAC	2880
2881	CAGTAGGCATGGACTCTCACTTCCAATCTGGCATTCTTCCCCACAG GGGCCCCTGGCAT	2940
2941	CCGGACAGGCATCCAAG CCCTTAAAGGAGACAGGGGGAACCT GGGCCCCTTGGAACCC	3000
3001	CGGCAAGGTGGGCTACCA GGGCCC AGCGGCCCTTCGG GGCCCTGGCATCCCGGAAT	3060
3061	TAAAGGCACCAAGGGCAGCCAGGAAACATCAAGGACCAGCCGAGGCCAGCCTTCTCCGC	3120
3121	CATTCCGGCGGAACCCCCCAATGGGGGGCAACGTGGTCATCTTCGACACGGTCATCACCAA	3180
3181	CCAGGAAGAACCCTACCAAGAACCACTCCGGCCGATTCTGCTGCACTGTACCCGGCTACTA	3240
3241	CTACTTCACCTTCCAGGTGCTGTCCAGTGGGAAATCTGCCT GTCCATCGTCTCCTCCTC	3300
3301	AAGGGCCAGGTCCGACGCTCCCTGGGCTTCTGTGACACCACCAACAAGGGGCTCTTCCA	3360
3361	GGTGGTGTACGGGGGCTGGTGTCTTCAGTCTCAGCAGGGTGACCAAGGTCTGGGTTGAAAA	3420
3421	AGACCCCAAAAAGGTCACATTTACCAAGGGCTCTGAGGCCGACAGCGTCTTCAGCGGCTT	3480
3481	CCTCATCTTCCCATCTGCCTGAGCCAGGGAAGGACCCCTCCCCACCCACCTCTCTGGC	3540

3541 TTCCATGCTCCGCCTGTAAATGGGGGCGCTATTGCTTCAGCTGCTGAAGGGAGGGGGCT 3600
 3601 GGCTCTGAGAGCCCCAGGACTGGCTGCCCCGTGACACATGCTCTAAGAAGCTCGTTTC TT 3660
 3661 AGACCTCTTCCTGGAATAAACATCTGTGTCTGTGTCTGTGTACATGAGCTTCAGTTGCT 3720
 3721 ACTCGGAGCATTGAGAGGGAGGCCTAAGAATAATAACAATCCAGTGCTTAAGAGTCAGGC 3780
 3781 CCGTCCTTAGTATGTTTTTTTGTGTTTTTTGTTTTTTAAGATGGAGTTTCTCTCTTGTCAAC 3840
 3841 CAGGCTGGAGTGCAATGGAGTGATCTTCGGTCACTGCAACCTCCGCCTCCCGGGTTCAAG 3900
 3901 CAACTCTCCTGCCTCAGTCTCCAGGTAGCTGGGATTACAGGTACCTGCCACCATGCCCC 3960
 3961 GTGAATTTTTTGTATTTTTTAGTAGAGAAGGGGTTTCACCATGTTGGCCAGGCTGGTCTCG 4020
 4021 AACTCCTGACCTCAAGTGATCCTCCAGCCTTGGCCTCCCAAAGTGCTGGGATTAGAGGCA 4080
 4081 TGTGCCACCACGCCCAGCCCATTCTTAGTATTTACATGATGTGTTTAGCATGTCTTAGC 4140
 4141 AGCCTCATCTACCCAGGTTCTGAATCCTGATGCCACCACTTATTAGCTGTCTAATCATGTG 4200
 4201 CATGTTAACCAGCTCTGAGATCTCAGTTTCCTCATCTGTAAAGTGGGACTGATGATCCC 4260
 4261 AAAGGGTCATTGGGAAGATTAAATGACTTAATATATGAAAATT





.. SNP G→A (no amino acid change: Gly)
 Original outer primers
 Original inner primers
 Extensions to inner primers used by Cartron as single primers (unpublished)
 GGGCC⁺C Potential *Apa*1 restriction sites

Appendix 9: C3 gene sequence – Selected section showing primer annealing and polymorphic sites.

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12241 GAGACTCCTCCCCCTTCTGAGTCCCTCCCTTCCCTGAGACCCACCCCTTCTGAGGTTCTCT 12300
12301 CCCCTTCTCTGAGACTCCACCCCTTCTGAGTCTCCTCCCCCTCTAAGTCCCTCCCACTGA 12360
12361 ATTCCTTTTCCAAGCCCTCCCCCTCGAAGTCTCCTTCTGAACTCCTCCCCCTCTTAGT 12420
12421 CTCCATCACTTTCTAAGTTCCTCACCTGAGTCCCTCCCCCTTCTGAGCCCTCCCATG 12480
12481 TCAGCCCTTCCCTTTCTGAGTCCCGCCCTTCTGAGCCCTCCTCCTATAAGCTCTCT 12540
12541 CCTCTTGTGAGCTCTTCTTTTGTGAGTTCCTTCCCTGGTCCCCCTCTCCCTCGCACCT 12600
12601 CCTTCACATGCCCTCCCTCCCAAACCGGCCACCTCGGAAAGACCAAGAATAATGGGCAG 12660
12661 GCAAGGAGGGACCCAGCCCAAGATCCGGAAGCTGGACCGTGGGCATGGGGCCTTGGAAACA 12720
12721 AACCCTTGACAATGCCCTGCCACGCCTAGATCCAGCCAACAGGGAGTTCAAGTCAGAA 12780
12781 AAGGGCGCGCAACAAGTTGGTGACCGTGCAGGCCACCTTCGGGACCCAAGTGGTGGAGAAG 12840
12841 GTGGTGCTGGTCAGCCTGCAGAGCGGGTACCTCTTCATCCAGACAGACAAGACCATCTAC 12900
12901 ACCCTTGGCTCCACAGGTGAGGCTGGGGGCGGCTGGAGAGGGCGGGGCACCGCGTGGGC 12960
12961 GGGCTAGGGTCTCAGGAGGCTCTTGTCTCTCCCCAGTTCTCTATCGGATCTTCACCGT 13020
13021 CAACCACAAGCTCTACCCGTGGGCGGACGTCATGGTCAACATTGAGGTGCCAGCCAG 13080
13081 AGGGGGCCCCAGGGGAAGCAGGGGCACAGGCTTAGGAGAGGCCAAGAGATCGAGAGAGAC 13140
13141 AGAGAAAGACACACCGGAAGGGGTGCAGTGGCAGAGACACAGAGGCAAAGAGATATGCAG 13200
13201 ACACACACCCACACAACACACACACATACAGCACACAACATGCACACACACAGCACACAA 13260
13261 TACACACACAGAGGCAAAGAGATATGCAGACACATGTGCACACACAATGCACACACACAA 13320
13321 TGCAACACACACAAACACACAACATACAGACCACACAACACACAACACAACACACAA 13380
13381 CACAATACACACAGCACAAACGTGCATGACCACACACAACACACAACACACAACACACA 13440
13441 ATACACAACATACACAACCACGCAATACACAAAAACACACAACACAACACACATAC 13500

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 SNP C→G (predicting Arg→Gly)
 published primers
 unpublished primers
 Potential *Hha*I restriction site

Appendix 10: Reagents

EDTA (Ethylenediaminetetraacetic acid) – a ligand and chelating agent which sequesters metal ions in solution, such that they are much less reactive.

TBE (Tris/Borate/EDTA) - a buffer solution containing a mixture of Tris (trishydroxymethylaminomethane; $(\text{HOCH}_2)_3\text{CNH}_2$), boric acid (H_3BO_3) and EDTA (ethylene diamine tetraacetic acid; $(\text{HO}_2\text{CCH}_2)_2\text{NCH}_2\text{CH}_2\text{N}(\text{CH}_2\text{CO}_2\text{H})_2$). Tris-acid solutions are effective buffers for slightly basic conditions, which keep DNA deprotonated and soluble in water. EDTA is a chelator of divalent cations, which are necessary co-factors for many enzymes.

Procedure: Add 9.3 g EDTA, 55 g Boric Acid and 108 g Tris to 1L ddH₂O. Mix with a magnetic stirrer for about 10 minutes then put in a 10 L tank with a further 9 L of ddH₂O.

DTAB (Dodecyltrimethylammonium bromide) – widely used lysing reagent

Trizol is a mono-phasic solution of phenol and guanidine isothiocyanate which disrupts cells and dissolves cellular components whilst maintaining the integrity of RNA.

Chloroform

Ethanol

Taq 2x - QIAquick Kits contain a silica membrane assembly for binding of DNA in high-salt buffer and elution with low-salt buffer or water. The purification procedure removes primers, nucleotides, enzymes, mineral oil, salts, agarose, ethidium bromide, and other impurities from DNA samples

X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) is an organic compound consisting of galactose linked to a substituted indole. It is used test for the presence of an enzyme, β -galactosidase. Bacteria which express β -galactosidase cleave X-Gal, leading to a colour change

Appendix 11: Results of *FCGR3A*-V158F polymorphism testing in patients randomised to arm A (watch and wait).

The 87 patients were ranked according to assessed tumour bulk at baseline. The number in each quartile for tumour bulk is presented here, with Q1 representing lowest bulk through to Q4 representing highest bulk.

Polymorphism result in 87 patients not treated with rituximab			
Tumour bulk	FF (n=39)	VF (n=38)	VV (n=10)
Q1	10 (26%)	10 (26%)	2 (20%)
Q2	12 (31%)	8 (21%)	2 (20%)
Q3	8 (20%)	11 (29%)	3 (30%)
Q4	9 (23%)	9 (24%)	3 (30%)